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REVIEW ARTICLE

SEMINARS IN MEDICINE
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JEFFREY S. FLIER, M.D., *Editor*
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THE TUMOR NECROSIS FACTOR LIGAND AND RECEPTOR FAMILIES

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TUMOR necrosis factor (TNF) and lymphotoxin-α were isolated more than 10 years ago, on the basis of their ability to kill tumor cells in vitro and to cause hemorrhagic necrosis of transplantable tumors in mice.[1] The complementary DNAs and genes encoding each protein were cloned immediately thereafter.[2,3] Concurrently, a factor known as cachectin was isolated from mouse macrophages, sequenced, and shown to be identical to TNF.[4,5] Cachectin was identified not as a cytolysin, but as a catabolic hormone that suppressed the expression of lipoprotein lipase and other anabolic enzymes in fat.[6-8] Still other studies demonstrated the powerful pro-inflammatory effects of TNF[9,10] and revealed its role as a central endogenous mediator of endotoxic shock.[11,12] Hence, TNF has a broad spectrum of biologic activities.

Because it proved to be highly toxic in animals and humans, TNF did not fulfill initial expectations that it would be useful in the treatment of cancer. However, considerable evidence suggests that overproduction or inappropriate production of TNF may play a part in various chronic inflammatory diseases. Produced largely by macrophages in response to inflammatory stimuli such as lipopolysaccharide, TNF binds to receptors present on virtually all cells throughout the body. TNF, if released systemically in large amounts all at once, modifies the anticoagulant properties of endothelial cells, activates neutrophils, and induces the release of other inflammatory cytokines. These effects culminate in cardiovascular collapse. By contrast, chronic, low-level production of TNF may contribute to the inflammatory response. Bone resorption, fever, anemia, and wasting may all, in some measure, be attributable to TNF[13] (Fig. 1).

What beneficial functions does TNF have? Can its activities be blocked, and if so, at what risk? Above all, how does TNF work? Answers to each of these questions have begun to emerge. They point to regulatory mechanisms that control the biosynthesis of TNF, address the molecular reactions that permit TNF to mediate cell signaling, and suggest a practical means of blocking the activity of TNF for therapeutic effect.

THE TNF-LIGAND AND TNF-RECEPTOR FAMILIES

TNF is one of 10 known members of a family of ligands that activate a corresponding family of structurally related receptors (Table 1). The receptors initiate signals for cell proliferation and apoptosis (programmed cell death). These signals are required for the normal development and function of the immune system. Excessive signaling through some of the receptors can cause severe inflammatory reactions, tissue injury, and shock. Mutations of the genes corresponding to the ligands or the receptors can cause characteristic disturbances of lymphocytes, derangement of the immune response, or autoimmune disease.

All members of the TNF-ligand family are believed to consist of three polypeptide chains. All but lymphotoxin-β (which consists of a single lymphotoxin-α subunit and two lymphotoxin-β subunits) are made up of three identical subunits. All except lymphotoxin-α (which is entirely secreted) and TNF (which is predominantly secreted) are transmembrane proteins that act chiefly through cell-to-cell contact. Nerve growth factor, a dimeric protein, is not actually a member of the TNF-ligand family. Rather, it was apparently adapted in the course of evolution to serve its receptor, a true member of the TNF-receptor family.[33]

All members of the TNF-receptor family are believed to be transmembrane proteins that consist of two identical subunits. The family is defined by a cysteine-rich amino-acid motif that recurs three to six times in the extracellular domain. The cytoplasmic domains vary more than the extracellular domains. Notably, certain receptors contain a 60-residue cytoplasmic sequence known as the "death domain." In the 55-kd TNF receptor and the Fas receptor, this domain is required for the transduction of an apoptotic signal.

With the exceptions of TNF and lymphotoxin-α, each member of the ligand family binds to a specific receptor. TNF and lymphotoxin-α engage two receptors (the 55-kd and 75-kd TNF receptors) with similar affinity. These two cytokines initiate similar (if not identical) biologic responses, although they are produced by different types of cells (lymphotoxin-α is produced exclusively by lymphocytes and natural killer cells, and TNF predominantly by macrophages) in response to different stimuli (antigenic or mitogenic stimuli for lympho-

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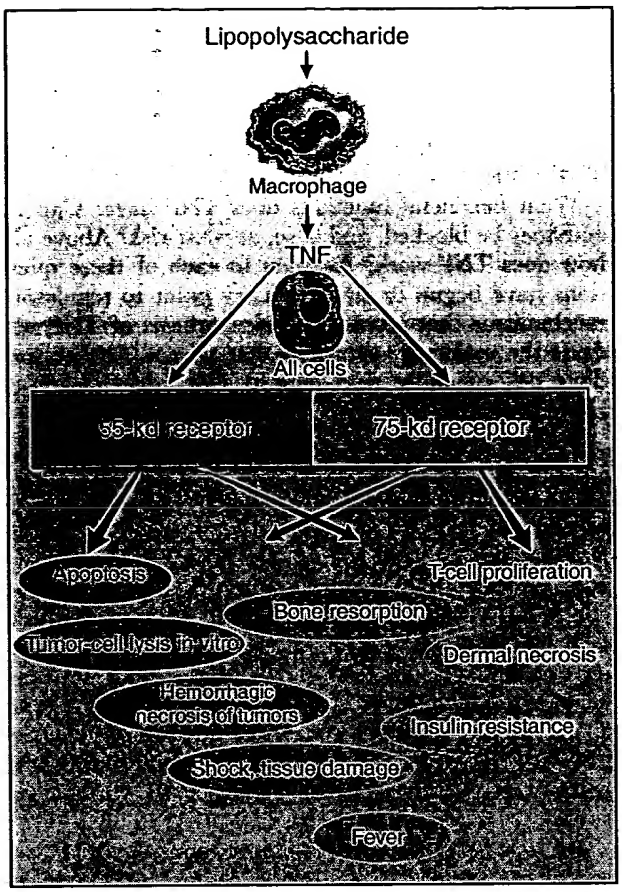

Figure 1. Range of Actions of TNF.
In response to inflammatory stimuli such as lipopolysaccharide, macrophages produce TNF. TNF binds to receptors present on virtually all cells throughout the body, causing a variety of reactions.

toxin-α and lipopolysaccharide or other macrophage-activating agents for TNF).

Functions of TNF Ligands and Receptors

For many years, the role of members of the TNF-ligand and TNF-receptor families in immunity and in the development of the immune system remained speculative. The first indication of their function came from the finding that mutations of the ligands or their receptors can cause disease. Striking examples are the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mutations of mice, which were found to specify defects of the Fas receptor (*lpr*) and the Fas ligand (*gld*). These mutant mice have long been taken as models of systemic lupus erythematosus because they have lymphadenopathy and splenomegaly and form autoantibodies.[34,35] In the homozygous state, each mutation causes the accumulation of large numbers of T cells lacking the CD4 and CD8 surface proteins. Heterozygous pairing of the lpr^{CG} allele (CG denotes "complements *gld*") with the *gld* allele also causes lymphoproliferation, suggesting that the products of the two loci might interact with one another.[36]

Further study demonstrated that the lpr^{CG} allele encodes a mutant Fas receptor that can bind its ligand but lacks signal-transducing activity. The classic *lpr* mutation, by contrast, abolishes the expression of the Fas receptor.[37-39] The lpr^{CG} allele contains a point mutation within the death domain of the Fas receptor that prevents signal transduction but does not affect ligand binding.[38] The *gld* allele contains a point mutation that inactivates the Fas ligand.[40] It is widely suspected that mutations of the Fas ligand or its receptor cause a failure of apoptosis in T lymphocytes. This, in turn, leads to massive accumulations of lymphocytes in lymph nodes and spleen and features of autoimmunity.

Humans with mutations of the Fas-receptor gene have also been identified. As in mice with similar mutations, these patients have lymphadenopathy, splenomegaly, and signs of autoimmunity at an early age.[41,42] Mutations of the Fas-receptor gene in humans with lymphoproliferative disease may be associated with the lack of a receptor protein; missense mutations of the gene interfere with signal transduction by the receptor because of faulty interaction with downstream signaling components.

Other members of the TNF-ligand and TNF-receptor families are also important in immune function. For example, the syndrome of X-linked immunodeficiency, in which there are high levels of IgM and low or absent levels of other immunoglobulins, is caused by a mutation in the CD40 ligand.[29,30] Interaction of the CD40 ligand on T cells with the CD40 receptor on B cells mediates immunoglobulin-class switching (the conversion from the production of IgM antibodies to the production of IgG antibodies) and clonal expansion of antigen-responsive B cells. In mice, deletion of the CD40-ligand or CD40-receptor genes results in a phenotype that resembles the disease that occurs in humans.[31,32]

Since naturally occurring mutations that interfere with the function of TNF, lymphotoxin-α, or their two receptors have not been identified, it has been necessary to ablate the genes in mice through a "gene knockout." Both the 55-kd and the 75-kd TNF receptors have been deleted in this manner. Genetically engineered mice lacking the 55-kd TNF receptor are moderately resistant to the lethal effect of lipopolysaccharide but highly susceptible to infection by *Listeria monocytogenes*.[18,19] Mice lacking the 75-kd TNF receptor are moderately resistant to the lethal effect of TNF itself and to dermal necrosis elicited by repeated intradermal injections of TNF.[21] Animals lacking both receptor genes have the sum of these phenotypic effects but no gross developmental effects. It would thus appear that, consistent with the different structure of their cytoplasmic domains, the two TNF receptors fulfill different functions in vivo.

Surprisingly, deletion of both the TNF and lymphotoxin-α genes, which removes the only ligands known to interact with the two TNF receptors, does not yield the same phenotype as the deletion of both receptors. On the contrary, mice in which the lymphotoxin-α gene has been deleted, either alone or in combination with the TNF gene, have no lymph nodes and Peyer's patches and no splenic white pulp. The thymus is grossly preserved.[17]

Table 1. Recently Characterized Members of the TNF-Ligand and TNF-Receptor Families.*

Ligand	Source of Ligand	Receptor	Distribution of Receptors	Ability to Initiate Apoptosis	Cytoplasmic Mediators	Mutation or Knockout Phenotype	
						Ligand	Receptor
TNF and lymphotoxin-α	TNF: macrophages, lymphocytes, keratinocytes, others	55-kd TNF receptor	Many cells	Yes (strong)	TRADD,[14] TRAP-1,[15] 55.11[16]	Both TNF and lymphotoxin-α: absent lymph nodes, decreased lipopolysaccharide responses	Decreased lipopolysaccharide responses; failure to contain listeria or mycobacteria infection[17,18]
	T cells	75-kd TNF receptor	Many cells	Yes	TRAF-1, TRAF-2[19]	Lymphotoxin-α: absent lymph nodes[20]	Decreased lymphocyte proliferation; decreased dermal responses to TNF; decreased TNF-induced lethality[21]
Lymphotoxin-β heteromer	T cells, others	Lymphotoxin-β receptor (TNF-receptor–related protein)	T cells, B cells, others	Yes	LAP-1 (CRAF-1)[22]	ND	ND
Fas ligand	T cells	Fas receptor	Many cells	Yes (strong)	Tyrosine phosphatase (FAP-1),[23] FADD (MORT-1)[24]	Lymphoproliferation	Lymphoproliferation
Nerve growth factor	NA	Nerve growth factor receptor	Neurons, others	No	ND	NA	Neuropathy[25]
CD40 ligand	T cells	Nerve growth factor receptor	B cells, T cells	No	CRAF-1, CAP-1[26-28]	X-linked immunodeficiency with increased IgM and decreased or absent IgG, IgA, IgD[29-31]	X-linked immunodeficiency with increased IgM and decreased or absent IgG, IgA, IgD[32]
CD27 ligand	T cells	CD27	T cells	ND	ND	ND	ND
CD30 ligand	T cells	CD30	T cells	ND	ND	ND	ND
OX-40 ligand	T cells	OX40	T cells	ND	ND	ND	ND
4-1BB ligand	T cells	4-1BB	T cells	ND	ND	ND	ND

*Other members of these two families include the CD27, CD30, OX-40, and 4-1BB ligands and receptors. TRADD denotes TNF-receptor–associated death domain, TRAP-1 TNF-receptor–associated protein 1, TRAF TNF-receptor–associated factor, LAP-1 latent membrane protein type 1–associated protein, CRAF-1 CD40-receptor–associated factor 1, ND not determined, FAP-1 Fas-associated protein 1, FADD (or MORT-1) Fas-associated death domain, NA not applicable, and CAP-1 CD40-associated protein 1.

These results may reflect the fact that the lymphotoxin-α gene is required for the formation of not only the lymphotoxin-α homotrimer but also the lymphotoxin-β heteromer.[43] Interaction between the lymphotoxin-β heteromer and the lymphotoxin-β receptor is probably required for lymph-node development.[17,44,45] Lymph nodes fail to develop in lymphotoxin-α–deficient mice despite the fact that the lymphoctye subtypes are distributed normally in peripheral blood. Lymphotoxin-α–deficient lymphocytes are readily incorporated into the lymph nodes of normal mice, and lymphotoxin-α–positive lymphocytes fail to induce the development of lymph nodes in lymphotoxin-α–deficient mice. Therefore, the failure of lymphotoxin-β heteromer to be expressed on the surface of a nonlymphoid cell, such as a lymph-node stromal element, would seem to preclude normal development of peripheral lymphoid tissues.[46]

How the Receptors Work

Interactions between TNF and its receptors are presumably typical of interactions between the other ligand–receptor pairs in these two molecular families. Trimeric ligands of the TNF family may cause aggregation or clustering of receptor subunits, thereby triggering a cellular response, since antibodies against either of the two TNF receptors mimic the actions of TNF.[47,48] Support for the aggregation model (Fig. 2A) is bolstered by the crystal structure of lymphotoxin-α, which forms a complex with extracellular-domain fragments of the 55-kd TNF receptor,[49] in which three receptor fragments crystallize with each lymphotoxin-α trimer.

Crystallization of the extracellular domain of the 55-kd receptor in the absence of ligand yields a dimeric protein, in which each subunit is arranged head to head with the other. The 55-kd receptor on the cell surface might thus be dimeric in the absence of TNF. A dimeric conformation would allow for a hexagonal array of dimeric receptors and trimeric ligand molecules, which could generate signals by making contacts between their cytoplasmic domains (Fig. 2B). Alternatively, each dimeric receptor might act as an independent "molecular switch," undergoing rearrangement after engaging the trimeric ligand[50] (Fig. 2C).

The molecular-switch model is strongly supported by the effect produced by substituting an erythropoietin-receptor extracellular domain for the extracellular domain of either TNF receptor. This leads to constitutive signaling activity in the cell (i.e., a perpetual "on" state).[50] The conformation of the receptor dimer, rather than dimerization itself, is thus the critical issue in signal transduction. Moreover, an essential attribute of the extracellular domain of the TNF receptor is its ability to prevent signal transduction in the absence of a ligand. If the extracellular domains are removed,[51] grossly modified,[50] or displaced by an antibody,[47,48] signaling is initiated.

One of the most intriguing actions of TNF is the induction of apoptosis. Apoptosis is almost certainly relevant to some of the toxic effects of TNF, such as shock

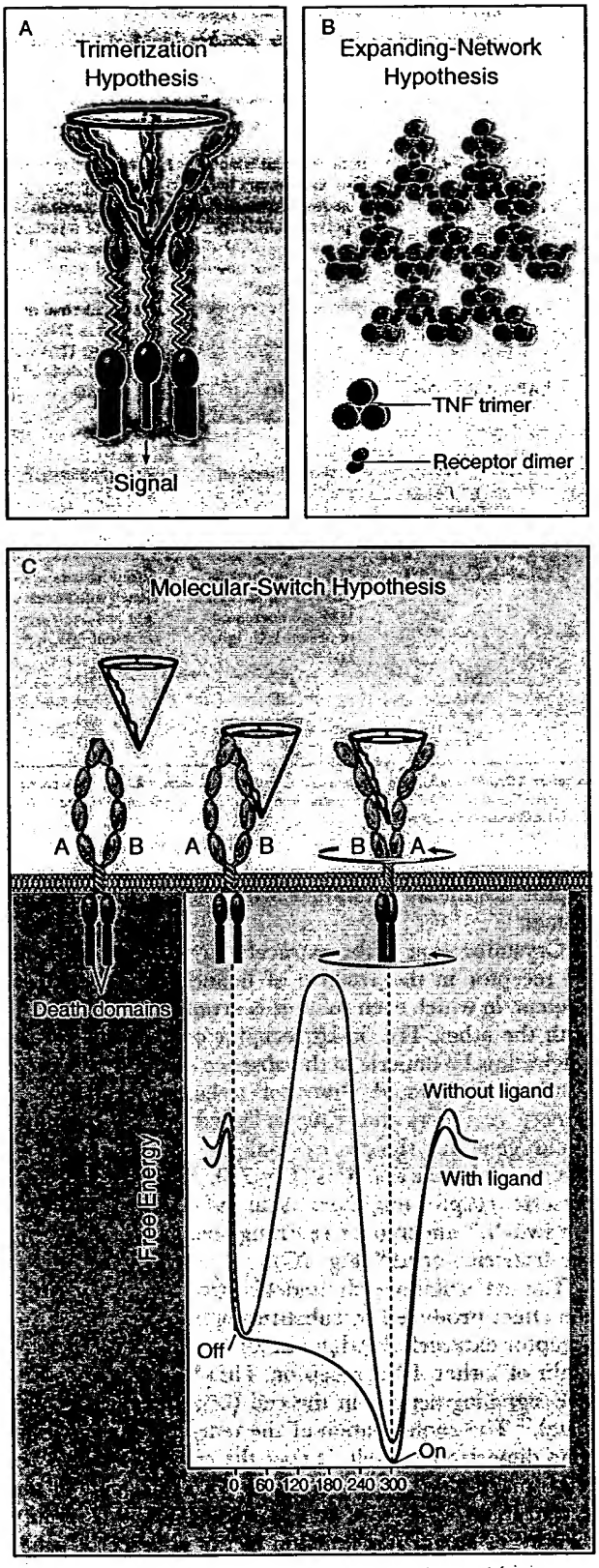

Figure 2. Three Models of the Molecular Events in TNF Signaling.

Panel A shows the trimerization hypothesis. In this model, the juxtaposition of three receptors results from their binding of a single TNF trimer. The resultant complex generates an activating signal.

Panel B shows the expanding-network hypothesis. A growing hexagonal array of TNF trimers bound to TNF-receptor dimers takes account of the dimeric structure of TNF receptors free of ligands and the capacity of each ligand molecule to engage three receptor subunits. "Capping" of receptors would trigger a biologic response.

Panel C shows the molecular-switch hypothesis, the most favored model. In this model each receptor dimer is an activatable unit. Receptor activation occurs in response to two events. First, the ligand binds to subunit B of the receptor. Second, subunit A disengages from subunit B, which permits binding of the receptor to a second available site on the ligand surface. These events cause conformational changes within the cytoplasmic domain of the receptor, leading to signal transduction. Specifically, the death domains of the 55-kd TNF receptor or Fas receptor might undergo homodimerization. The graph shows an imaginary profile of the free energy associated with conformational changes between the "off" and "on" states of the receptor. It is supposed that a large activation-energy barrier prevents transition from the off to the on state in the absence of ligand and that TNF effectively catalyzes this transition, thereby "throwing the switch." Moreover, as depicted here, the free energy of the on state, which presumes a stable association between TNF and the receptor, may be substantially lower than the free energy of the off state, and this may be irreversible.

and inflammation. TNF-induced apoptosis may also have physiologic relevance, as does the apoptosis induced by the Fas receptor. It is possible, for example, that TNF-mediated apoptosis of infected cells helps protect the host. It is likely that both TNF receptors participate in cell death, although the 55-kd receptor is more potent than the 75-kd receptor. Since the cytoplasmic domains of the two receptors are structurally different, each must initiate apoptosis through distinct mechanisms.

Proteins Used by the TNF-Receptor Family for Signal Transduction

Several proteins that bind intracellularly to receptors of the TNF family have been identified (Fig. 3). The first to be identified were the TNF-receptor–associated factors (TRAFs), which have high affinity for the 75-kd TNF receptor.[20] Their biologic function is unknown. Most contain two protein motifs termed the "zinc finger" and "ring finger." TRAF-2 may form a homodimer with itself or a heterodimer with TRAF-1. TRAF-2, TRAF-3 (also known as CD40-receptor–associated factor 1 [CRAF-1] or latent membrane protein type 1–associated protein [LAP-1]), and the closely similar CD40-associated protein 1 (CAP-1) bind not only to the 75-kd TNF receptor, but also to the lymphotoxin-β receptor and the CD40 receptor. Moreover, TRAF-3 also binds to latent membrane protein type 1, a protein of the Epstein–Barr virus that is essential to cell transformation.[22]

An entirely different class of cytoplasmic proteins bind to the 55-kd TNF receptor and the Fas receptor. These proteins are important in transducing signals for programmed cell death. The Fas-associated death domain (FADD),[52] also called MORT-1,[24] the TNF-receptor–associated death domain (TRADD),[14] and the receptor interacting protein (RIP)[53] bind to the Fas receptor, the 55-kd TNF receptor, and both receptors, respectively. Each of these proteins contains a version of the death domain found within the receptors them-

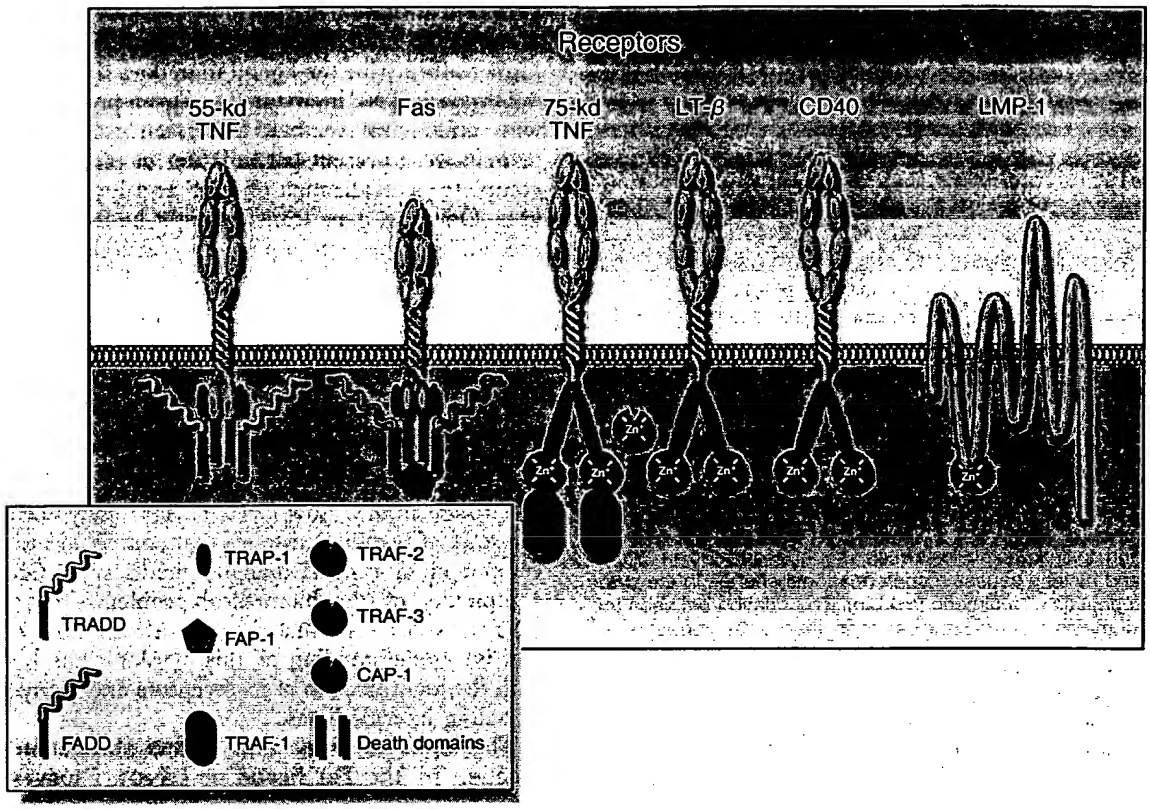

Figure 3. Proteins That Bind the Cytoplasmic Domains of Receptors for TNF, Lymphotoxin-α, Lymphotoxin-β (LT-β), CD40 Ligand, Fas Ligand, and Latent Membrane Protein Type 1 (LMP-1).

Lymphotoxin-α and TNF bind to the same 55-kd and 75-kd TNF receptors. Proteins and domains with similar structures or functions, such as TNF-receptor–associated death domain (TRADD), receptor-interacting protein (RIP), Fas-associated death domain (FADD, or MORT-1) molecules, and the death domains of the Fas and 55-kd TNF receptors, are generally portrayed by identical shapes but in different colors. Zn is used to denote proteins known to contain ring-finger and zinc-finger motifs that bind to the 75-kd TNF receptor, lymphotoxin-β receptor, and CD40-ligand receptor. TNF-receptor–associated factor type 1 (TRAF-1) lacks a zinc-finger motif but binds to TRAF-2, which has both ring-finger and zinc-finger motifs. CD40-associated protein 1 (CAP-1), which is structurally very similar to TRAF-3, is thought to be capable of replacing TRAF-3 as a binding partner for the lymphotoxin-β receptor and CD40-ligand receptor. LMP-1 is a plasma-membrane protein that spans multiple domains and is encoded by the genome of the Epstein–Barr virus. It has no homology to members of the TNF family. Not all possible combinations and interactions are shown. In addition, certain binding proteins that were not discussed in the text belong neither to the zinc- and ring-finger family nor to the death-domain family of transducers. These include TNF-receptor–associated protein 1 (TRAP-1), a heat-shock protein analogue[15]; the 55.11 protein, a proteasome regulatory subunit[16]; and Fas-associated protein 1 (FAP-1), a protein tyrosine phosphatase that binds to the Fas receptor near its carboxy terminal and is thought to decrease the intensity of signals generated by this receptor.[23]

selves. This motif permits interaction between receptor and transducer molecules.

FADD[24,52] is a proximal transducer of the apoptotic activity of the Fas receptor, with which it forms a heterodimer. Engagement of the ligand causes the release of homodimeric FADD, which relays the death signal to the cytoplasm. FADD is incapable of forming heterodimers with receptors encoded by the mutant *lpr*^{CG} gene. Hence, it is at precisely this level that the *lpr*^{CG} mutation interrupts signaling.

Acting in an analogous fashion, TRADD is a proximal transducer of apoptosis mediated through the 55-kd TNF receptor.[14] RIP appears to serve both the 55-kd TNF receptor and the Fas receptor. The transducers may interact with distinct targets downstream from the receptor. Although overexpression of any of the transducers can initiate cell death, different portions of each protein act to carry the apoptotic signal forward within the cytoplasm.[54] Moreover, RIP is far larger than either TRADD or FADD and contains a kinase domain.[54]

In accordance with the molecular-switch hypothesis, a receptor modified by the binding of ligand might effectively catalyze the formation of homodimeric FADD, TRADD, or RIP (Fig. 4), causing conformational changes in these molecules that lead to further reactions in the signaling cascade. This scenario is consistent with the observation that modified receptors are constitutively activated in the absence of ligand.[50] A detailed discussion of the distal components of the cascade by which the death signal is conveyed is beyond the scope of this review.[55-58]

Clinical Effects of TNF and Lymphotoxin-α Blockade

Extensive clinical trials have been performed to test monoclonal antibodies that selectively neutralize TNF (but not lymphotoxin-α) in the treatment of septic shock. To date, double-blind, controlled studies have not documented a substantial benefit.[59,60] However, septic shock is a fulminant disease in which considerable

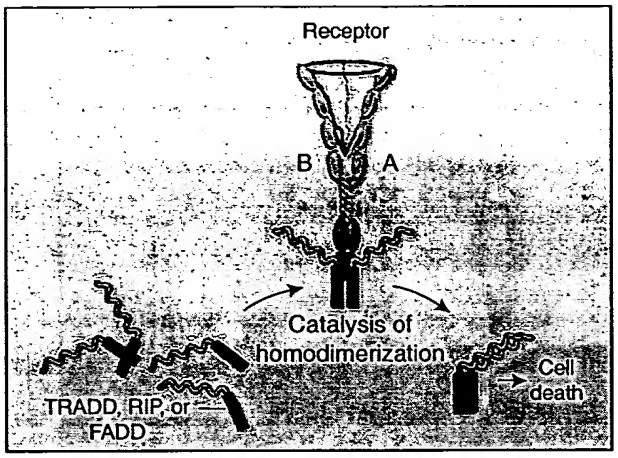

Figure 4. Proposed Catalysis of the Homodimerization of TRADD, RIP, and FADD by the Cytoplasmic Domain of Ligand-Activated Receptor.

According to the molecular-switch model presented in Figure 2C, ligand binding causes a conformational change in the cytoplasmic domain of a dimeric receptor. The relation between the death domains (red rectangles) then favors the formation of TRADD, RIP, or FADD (or MORT-1) homodimers, permitting signal transduction to be carried downstream to components of the pathway that results in cell death. Homodimers do not form spontaneously unless TRADD, RIP, or FADD is artificially expressed at very high levels in the cell. A and B refer to the subunits of the receptor.

damage may already have occurred before the initiation of therapy. The different goal of treating chronic inflammatory diseases, in which slow, continuous tissue damage may occur owing to the presence of TNF, has met with greater success. For example, anti-TNF antibodies lessen pain, joint swelling, anemia, and elevated erythrocyte sedimentation rates in patients with rheumatoid arthritis.[61-63] Encouraging results have also emerged from the use of anti-TNF antibodies in the treatment of Crohn's disease.[64]

Therapy with anti-TNF antibodies has several shortcomings, however. First, anti-TNF antibodies do not prevent signaling of either of the homotrimeric TNF receptors, since they do not neutralize lymphotoxin-α. Second, the antigenicity of murine monoclonal antibodies, and even humanized monoclonal antibodies, may preclude long-term therapy. Third, complement fixation and other reactions related to the formation of immune complexes might be harmful in patients receiving anti-TNF antibodies. Finally, the concentration of monoclonal anti-TNF antibody required to achieve neutralization is very high; consequently, blockade might be expensive to maintain.

The use of chimeric inhibitor molecules (Fig. 5), in which the extracellular domain of the TNF receptor is spliced to an immunoglobulin heavy-chain fragment, might circumvent all these problems. Such molecules are as stable in vivo as immunoglobulins. Because they are composed of two nonantigenic elements, they are minimally antigenic. Their mode of action is highly specific, since their binding domain is a receptor. Moreover, they are broad-spectrum agents, since they neutralize both TNF and lymphotoxin-α, preventing the activation of both TNF receptors. On a weight basis, chimeric inhibitors are far more potent than monoclonal anti-TNF antibodies, because the receptor has a far higher affinity for the ligand than does the antibody.[65,66]

Chimeric TNF inhibitors have been produced in mice with adenoviral vectors. Milligram quantities of the protein were present per milliliter of plasma,[67] causing complete neutralization of TNF and lymphotoxin-α in vivo. One day, gene transfer might be used to produce the inhibitor protein in patients with inflammatory diseases related to the overproduction of TNF.

The Future of TNF

The breadth of actions ascribed to TNF is remarkable (Fig. 1). The molecule is one of the best-characterized gateways to apoptosis. It is essential for defense against intracellular pathogens. It is a pro-inflammatory mediator that can, when overproduced, cause shock and tissue injury. Low levels of TNF may account for the state of insulin resistance that contributes to the development of type II diabetes mellitus.[68-75] Given the complexity of the biomedical problems that involve TNF, it is fortunate that the groundwork for a comprehensive understanding of this cytokine has been laid with the identification of its receptors and many of their signaling intermediates.

The lupus-like state that follows abrogation of the function of the Fas ligand or receptor suggests that some autoimmune disorders could involve defects in the Fas- or TNF-signaling axes. The observation that the administration of TNF attenuates or prevents some autoimmune diseases in animals supports this view.[76-79] TNF may never prove useful in the treatment of widely disseminated cancer, but the insight into tumor-cell vulnerability gained through studies of TNF signal transduction may ultimately yield novel chemotherapeutic approaches.

Ways to block the biosynthesis or action of TNF could have important clinical applications. TNF has served as the principal end point in most studies of endotoxin signal transduction. It is likely that drugs impairing each step of that process will soon be tested for antiinflammatory efficacy. TNF and lymphotoxin-α can already be neutralized, and neutralization of other members of the ligand family is being explored.[80] Thus, new and highly specific approaches to the treatment of inflammatory disease may soon be at hand.

Discussion

DR. JEFFREY FLIER: What is the relative role of transcription and translation in vivo in activating TNF? Can you discuss your work on transgenic mice that relates to this issue?

DR. BEUTLER: The biosynthesis of TNF is controlled by two switches — one transcriptional and one translational — that work in concert with each other. The activation of macrophages by lipopolysaccharide causes a 50-fold increase in TNF messenger RNA and a 100-fold increase in translational efficiency. The rate of production of TNF protein increases by a factor of several thousand. My colleagues and I created transgenic mice bearing a reporter construct in which the TNF coding sequence was replaced by DNA coding

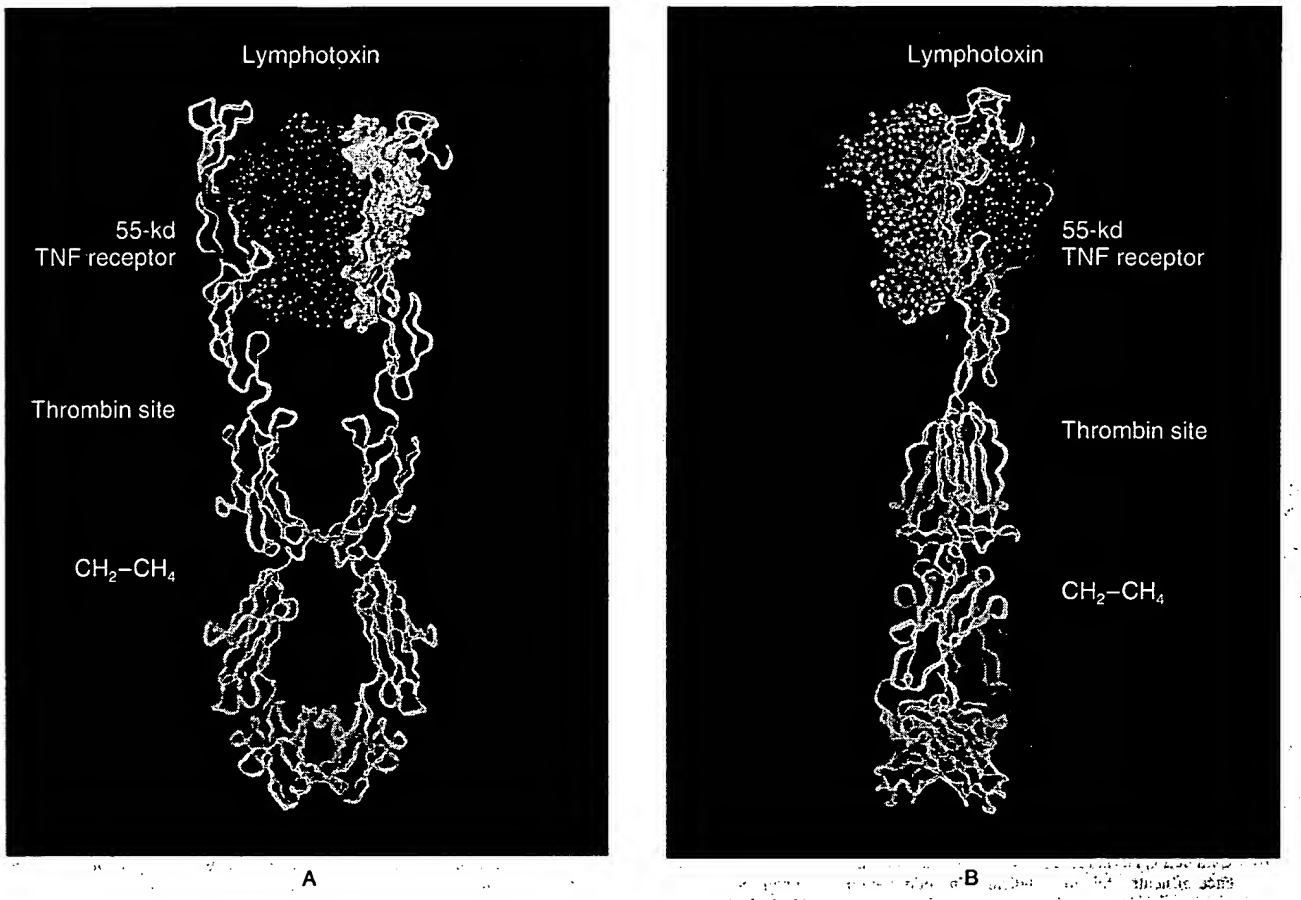

Figure 5. Predicted Tertiary and Quaternary Structures of TNF-Inhibitor Molecules Created by Fusing the Extracellular Domain of the TNF Receptor to Domains CH_2, CH_3, and CH_4 of an IgG Heavy Chain.

Two views of the molecule, binding to a lymphotoxin-α trimer, are shown (Panels A and B). The binding of lymphotoxin-α to the extracellular domain of the 55-kd TNF receptor was reported by Banner et al.[49] and is used to model this portion of the chimeric protein (yellow). The IgG moiety is blue. The lymphotoxin-α trimer subunits are green, white, and red. This model was assembled by Dr. Stephen Sprang.

for the marker enzyme chloramphenicol acetyltransferase, or CAT. The CAT-reporter transgene behaved rather like the TNF gene itself and revealed to us that TNF is produced constitutively in the thymus and trophoblast. However, its function in these tissues remains unknown.

A PHYSICIAN: What signals do the ring- and zinc-finger proteins convey?

DR. BEUTLER: There is a tendency to think that they may transduce mainly proliferative signals. The receptors to which they bind have largely proliferative functions. This is true of the 75-kd TNF receptor and the CD40-ligand receptor. It may also be true of the lymphotoxin-β receptor. Common transducers may therefore produce a common effect, in what amounts to cross-communication among the three. On the other hand, each receptor does have unique properties, and it is unlikely that any one of them can truly substitute for another.

A PHYSICIAN: What do we know about how TNF actually causes apoptosis? Specifically, what makes tumor cells so sensitive to it?

DR. BEUTLER: It is still not clear why the cells are so sensitive. Almost any kind of cell can be lysed by TNF in the presence of protein-synthesis inhibitors. The general thinking has been that tumor cells lack a short-lived protective factor that enables normal cells to escape the cytolytic effect of TNF. Members of the family of proteases containing interleukin-1β–converting enzyme have been implicated as downstream transducers in the TNF and Fas signaling pathways. Inhibitory members of the Bcl-2 family of proteins, such as Bak, may also be involved. However, a clear and complete biochemical pathway has yet to be established. Therefore, we cannot say precisely why certain tumor cells are sensitive to TNF, nor do we know what advantage this might confer.

DR. VIKAS SUKHATME: Is it possible to make TNF antagonists?

DR. BEUTLER: True antagonists have not yet been designed, although theoretically it should be possible to achieve this. A heteromeric form of TNF, with only one site capable of binding to the receptor, might fulfill such a function.

DR. FLIER: Could you tell us whether the pharmaceutical industry is using the TNF inhibitors that you developed?

DR. BEUTLER: The chimeric inhibitors have now been produced for clinical use by a number of companies. They are being tested for efficacy in the treatment

of rheumatoid arthritis. Already, proof of principle has been supplied by the striking remissions induced by anti-TNF antibodies. There is reason to believe that chimeric inhibitors will perform even better, given their activity against lymphotoxin-α as well as TNF, their extraordinary affinity for these ligands, and their relative lack of antigenicity.

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Etanercept: therapeutic use in patients with rheumatoid arthritis

Leslie Garrison, Neil D McDonnell

Abstract

Tumour necrosis factor (TNF) plays a central part in the pathophysiology of rheumatoid arthritis (RA). TNF initiates signal transduction by interacting with surface bound TNF receptors. Soluble tumour necrosis factor receptors (sTNFRs) act as natural inhibitors of TNF activity. Etanercept, recombinant p75 sTNFR:Fc fusion protein, has received approval from the US Food and Drug Administration for patients with RA and juvenile RA (JRA) who have failed treatment with at least one other drug. Etanercept has demonstrated excellent safety and efficacy in large scale, randomised, double blind, placebo controlled trials of patients with RA and JRA who are refractory to other disease modifying antirheumatic drugs. The therapeutic effects mediated by etanercept are rapid and sustained. Combining etanercept with methotrexate was found to be safe and more effective than treatment with methotrexate alone in the treatment of RA. These clinical findings demonstrate that etanercept can result in symptomatic improvement in patients with RA and JRA. Etanercept is an important new addition to the treatment of these diseases.

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Tumour necrosis factor (TNF) is known to play a central part in the pathogenesis of rheumatoid arthritis (RA). As the importance of TNF in RA began to be understood, the intriguing possibility emerged that blocking the activity of TNF might improve RA symptoms and perhaps even slow disease progression. In the past few years, biological response modifiers capable of neutralising TNF have been developed and tested in patients with RA. One of these agents, etanercept, has recently been approved by the US Food and Drug Administration (FDA) for patients with RA or juvenile rheumatoid arthritis (JRA) who have failed treatment with at least one other disease modifying drug.

TNF plays a central part in the pathophysiology of RA

TNF is a proinflammatory cytokine that is primarily produced by monocytes, macrophages, and lymphocytes.[1–3] Bioactive soluble TNF is a homotrimer composed of three identical 17 kDa TNF molecules.[4] TNF must engage two or more membrane bound TNFRs to initiate intracellular signal transduction.[3]

The role of TNF in arthritis has been shown in mice transgenic for human TNF, in which synovitis and destructive arthritis spontaneously develop.[5] Additionally, TNF is found in high concentrations in the synovium and synovial fluid of patients with RA[2 6–11]; the level of TNF in the synovium correlates with the degree of synovitis and bone erosions.[6 12]

TNF triggers several important events that lead to the synovitis and tissue destruction exhibited in RA. It causes proliferation of synoviocytes, production of other proinflammatory cytokines such as interleukin 1 (IL1), interleukin 6 (IL6), and GM-CSF; induces production of metalloproteinases such as collagenase and stromelysin, and increases expression of adhesion molecules. In addition, prostaglandin E_2 production by synoviocytes is increased, which, in conjunction with IL1, IL6, and TNF itself, increase proliferation and activity of osteoclasts, leading to destruction of bone.[13–16]

In addition to TNF, other proinflammatory cytokines, most importantly IL1, are thought to be involved in the pathogenesis of RA. As previously mentioned, TNF induces the expression of both IL1 and IL6.[17–19] In contrast, IL1 stimulates IL6 production, but seems to be a less potent stimulator of TNF production.[18]

Soluble TNF receptors: natural TNF inhibitors

Although the concept of blocking TNF activity has been considered since the mid-1980s, the discovery that membrane associated TNFRs existed in soluble forms that retained ligand binding capacity led to the relatively recent development of this novel approach for neutralising TNF. sTNFR based agents have several advantages as a means of inhibiting TNF activity. sTNFR based compounds do not require non-human amino acid sequences. This reduces their antigenic potential and minimises the likelihood that humans treated with these agents will develop antibodies against them that could potentially interfere with their therapeutic effects. Furthermore, sTNFR based agents effectively neutralise circulating TNF molecules. This distinguishes them from receptor antagonists, which prevent activity by interacting with the receptor but leave the active ligand in circulation.

There are two distinct TNF receptor molecules that bind TNF with comparable affinities, the p55 or type 1 TNFR and the p75 or type 2 TNFR.[20] Soluble forms of both receptors have been identified.[21] These sTNFRs can neutralise TNF activity both in vivo and in vitro, and both are believed to act as

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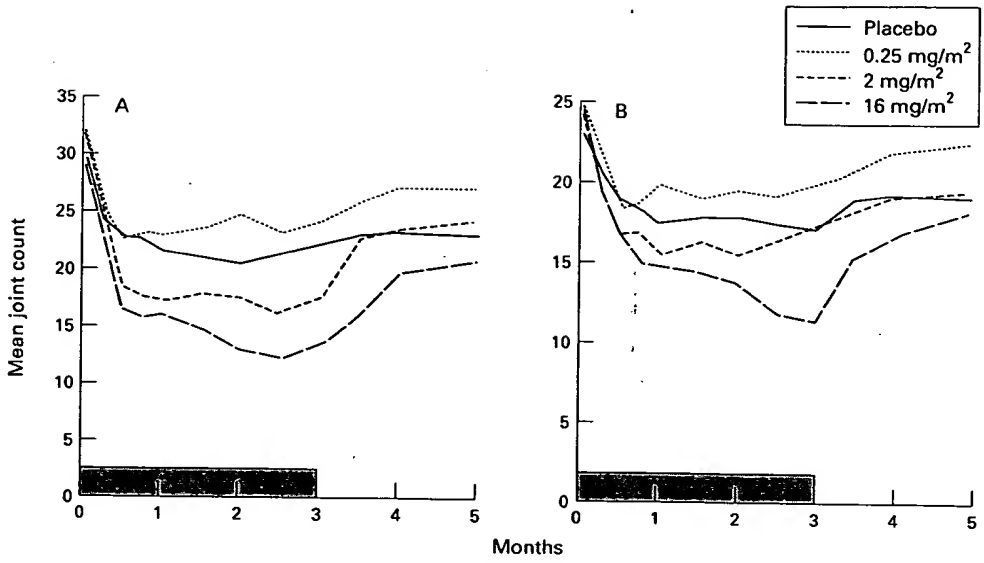

Figure 1 Effect of treatment on tender and swollen joint counts in phase II trial. (A) Mean tender joint count. (B) Mean swollen joint count. Shaded bars represent the treatment period.

natural inhibitors of TNF activity.[21-23] The levels of sTNFRs are often markedly increased in the sera and synovial fluid of RA patients.[23-25] However, it seems that an imbalance between TNF and sTNFR expression contributes to the perpetuation of inflammation and subsequent joint destruction in patients with RA.[23]

Development of etanercept for clinical use

Cloning of the TNFR genes has allowed the expression of recombinant forms of sTNFRs. Etanercept is a dimeric fusion protein consisting of the extracellular portion of the human p75 TNFR linked to the Fc portion of human lgG1.[26]

To better serve as therapeutic agents, etanercept was designed to improve upon the characteristics of monomeric sTNFRs. Like naturally occurring sTNFRs, it is highly specific for TNF and lymphotoxin α. However, compared with monomeric sTNFRs, etanercept has a much higher affinity for TNF.[26] In addition, the presence of the Fc portion of human IgG extends the half life of etanercept approximately fivefold to eightfold in vivo.

Clinical studies of sTNFR based agents in patients with RA

Etanercept has been assessed in clinical trials of adults with RA and patients with juvenile RA (JRA). On the basis of extensive data from the etanercept clinical trials, this agent became the first biological response modifier approved by the FDA for use in patients with RA, and JRA.

ETANERCEPT CLINICAL TRIALS

The potential clinical utility of etanercept in adults with RA was assessed in three placebo controlled, double blind, randomised clinical trials involving over 500 patients. In the first placebo controlled etanercept trial, a phase II trial involving 180 patients who had failed treatment with from one to four disease modifying anti-rheumatic drugs (DMARDs), patients received a subcutaneous injection of placebo or etanercept 0.25, 2, or 16 mg per square metre of body surface area) twice weekly for three months.[27] Etanercept produced a significantly greater improvement in all primary and secondary measures of disease activity than did placebo. A dose response relation was observed between etanercept dose and the primary outcome measures, with the greatest response from the 16 mg/m² dose (16 mg/m² is roughly equivalent to 25 mg). The mean reduction from baseline in total swollen and tender joint count was 61% with etanercept 16 mg/m² and 25% with placebo (fig 1). The difference between the two groups was apparent as early as two weeks after starting treatment. At the end of the three month treatment period, a significantly larger percentage of patients in the etanercept 16 mg/m² group (75%) than in the placebo group (14%) had at least a 20% improvement in the ACR. Similarly, 57% of patients receiving etanercept 16 mg/m² experienced a 50% improvement in symptoms, compared with only 7% of the placebo group (a significant difference). Disease activity returned toward baseline within two months after discontinuation of etanercept treatment, suggesting the need for continued treatment.

On the basis of the favourable results from the phase II study, a second placebo controlled study was conducted using a simplified etanercept dosing schema and an extended duration of treatment.[28] In this trial, 234 patients with refractory RA (inadequate response to 1–4 prior DMARDs) were randomised to receive placebo, etanercept 10 mg, or etanercept 25 mg subcutaneously twice weekly. All patients were required to have at least 12 tender/10 swollen joints, and at least one of the following: erythrocyte sedimentation rate (ESR) ≥ 28 mm 1st h, C reactive protein (CRP) > 2.0 mg/dl, or morning stiffness for at least 45 minutes. The primary efficacy end points were 20% and 50% improvement in disease activity at three and six months, as defined in the ACR

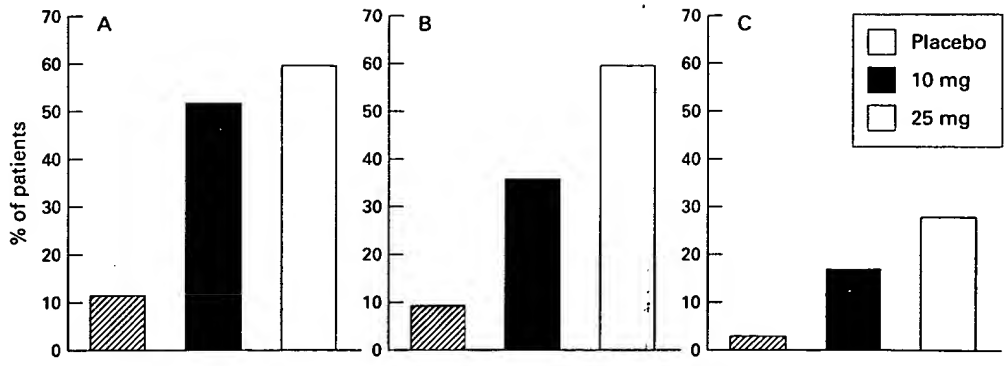

Figure 2 ACR responses at six months in phase III trial. (A) Per cent of patients achieving a 20% ACR response. (B) Per cent of patients achieving a 50% ACR response. (C) Per cent of patients achieving a 70% ACR response.

Table 1 Mean per cent improvement in quality of life at six months (Phase III)*

Variable	Placebo (n=80)	Etanercept 10 mg (n=76)	Etanercept 25 mg (n=78)
Disability Index	2	34†	39†
General health status	−12	34†	33†
Arthritis specific health status	−22	31†	44†
Vitality	2	22†	25†
Mental health	3	17	35†‡

*Positive numbers represent improvement, negative numbers represent worsening.
†p <0.05 for etanercept groups compared with placebo group.
‡p <0.05 for etanercept 10 mg compared with etanercept 25 mg.

response criteria. Secondary end points included the individual components of the ACR response. This trial employed joint assessors who were blinded to treatment, were not involved in patient care, and did not discuss the study with patients or investigators.

Etanercept produced significantly greater improvement in all primary and secondary measures of disease activity than did placebo, and a dose response was apparent, with etanercept 25 mg being more effective than etanercept 10 mg. At six months, 59% of patients in the etanercept 25 mg, 51% of patients in the etanercept 10 mg, and 11% of patients in the placebo group achieved a 20% ACR response ($p < 0.001$ for each etanercept group compared with the placebo group). The percentage of patients achieving a 50% and 70% ACR response was also superior in patients receiving etanercept (fig 2; $p \leq 0.001$ for etanercept 25 mg *v* placebo).

Clinical responses generally were observed within one or two weeks and nearly always within three months after starting treatment. Of patients receiving etanercept 25 mg, 15% experienced a 70% ACR response after three months and six months of treatment, compared with fewer than 5% of patients who received placebo. The components of patient assessment of physical function (that is, disability index) measured using the Health Assessment Questionnaire were improved to a significantly greater extent by etanercept than by placebo (table 1).

In the third randomised etanercept trial in adult RA, the combination of methotrexate (MTX) and etanercept was compared with MTX plus placebo.[29] In this double blind, randomised study, 89 patients with persistently active RA despite MTX treatment received subcutaneous injections of either placebo or etanercept 25 mg twice weekly in addition to continuing MTX (12.5 to 25 mg per week). The combination of etanercept and methotrexate resulted in 71% of patients achieving a 20% ACR response, compared with only 27% of patients who received methotrexate alone. As with the phase 3 trial, the percentage of patients achieving a 50% and 70% ACR response was statistically significantly greater for patients receiving etanercept (fig 3). Responses were rapid and durable.

The improvements in disease activity mediated by etanercept are maintained with long term treatment in the majority of patients (Moreland LW, *et al.* XIVth European Congress of Rheumatology, Glasgow, 1999). Of 51

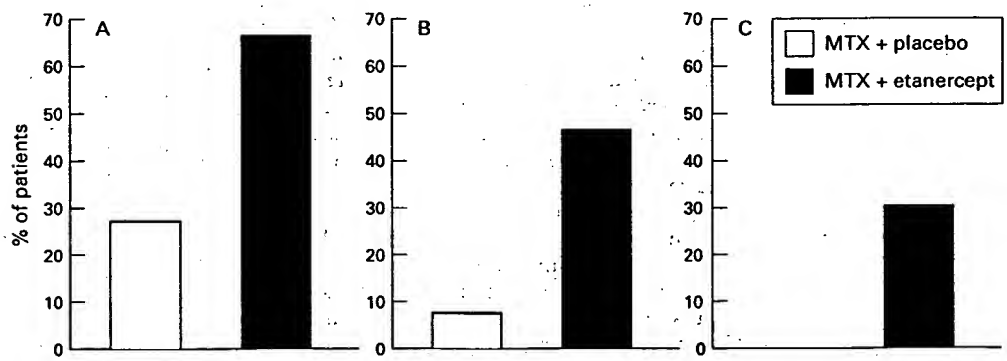

Figure 3 ACR responses at six months in methotrexate ± etanercept trial. (A) Per cent of patients achieving a 20% ACR response. (B) Per cent of patients achieving a 50% ACR response. (C) Per cent of patients achieving a 70% ACR response.

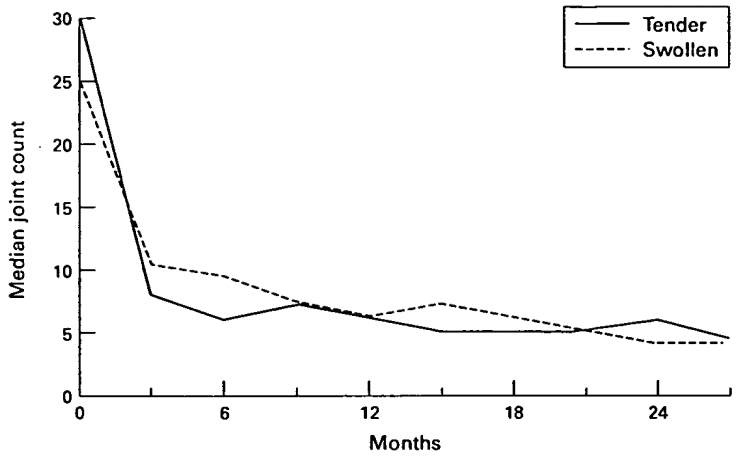

Figure 4 Median tender and swollen joint counts in long term open label etanercept trial.

patients treated with etanercept 25 mg subcutaneously twice weekly for 24 months in a long term open label trial, tender and swollen joint counts were improved by 87 and 84%, respectively, and 88% of patients achieved a Paulus 20 response at 24 months (fig 4).

Finally, preliminary results of a placebo controlled trial of etanercept in JRA have been reported (Lovell DJ, *et al.* American College of Rheumatology 62nd National Meeting, San Diego, 1998). Eligible patients were between the ages of 4–17 and had active, polyarticular course JRA. All patients received etanercept 0.4 mg/kg subcutaneously twice weekly for up to three months. Patients who met the JRA definition of improvement criteria at the end of three months were then randomised to continue on etanercept at the same dose and schedule or to receive placebo until disease flare occurred, or until four months elapsed, whichever was earlier. Sixty nine patients enrolled in the trial, and 51 (74%) met the criteria for improvement at three months and entered the blinded portion of the trial. A significantly greater number of patients who received placebo had disease flare than did patients who received etanercept.

Data from these studies indicate that etanercept has a favourable safety profile. In placebo controlled trials, the discontinuation rate attributable to adverse events was similar in the etanercept and placebo groups. The most common etanercept associated adverse event is injection site reaction, which developed in 37% of patients receiving etanercept versus 10% of those receiving placebo. This reaction is characterised by development of mild erythema, itching, pain, and/or swelling. The frequency of injection site reactions diminishes with time. In most RA patients these reactions do not necessitate drug discontinuation or require treatment.

Treatment with etanercept does not impair immunocompetence, as assessed by DTH skin testing, serum immunoglobulin concentrations, or enumeration of immune effector cell populations (Moreland LW, *et al.* American College of Rheumatology 62nd National Meeting). The overall incidence of infections and serious infections were similar in the etanercept and placebo groups in clinical trials. However, because data from clinical trials of etanercept in patients with microbial sepsis suggested an increase in mortality in patients receiving the highest dose (1.4 mg/kg body weight),[30] etanercept should be used with caution in patients with serious infections, and these patients should be closely monitored.

Anti-etanercept antibodies were detected at least once in the sera of 16% of treated RA patients at multiple time points. However, this positive titre generally appeared only sporadically, and the antibodies were non-neutralising. Development of antibodies did not correlate to clinical response or adverse events. Treatment with etanercept has no clear effect on the development of autoantibodies and, no patients developed clinical signs suggestive of a lupus-like syndrome or other new autoimmune diseases during clinical trials. Etanercept does not cause liver or kidney damage and thus regular blood monitoring is not required, and no increase in the incidence of malignancy has been observed to date in etanercept treated patients.

Future considerations

TNF antagonism with etanercept has already become an important new therapy for the treatment of RA and JRA. The experience obtained to date in clinical trials leaves little doubt about the efficacy of etanercept in controlling symptoms in these patients. The question remains about the ability of etanercept to slow disease progression. Based on the preclinical data on TNF, etanercept would be expected to slow bone and cartilage destruction; however, previous trials have not investigated this issue. This question will soon be answered when the results of the largest randomised trial to date of etanercept in RA are reported. In this study, patients with early RA (less than three years since diagnosis and no prior methotrexate treatment) were randomised to receive etanercept 10 mg, etanercept 25 mg, or optimal doses of methotrexate for 12 months. Patients were evaluated clinically, as in previous trials, and radiographically, to determine the extent of progression of bony erosions. The results of this trial are likely to have a major impact on the ultimate place for etanercept in the treatment of RA. In addition, as more experience with etanercept is gained, effects on corticosteroid, NSAID, and DMARD requirements will probably become available, as well as information about long term health impact such as effects on disability and requirements for joint replacements.

The success of etanercept in RA will probably be carried over into other TNF mediated diseases. Not only does TNF play a part in other rheumatological conditions but also in Crohn's disease, heart failure, and numerous other inflammatory conditions. Controlling TNF represents an important new avenue by which many diseases may one day be treated.

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The nerve growth factor/tumor necrosis factor receptor family

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Abstract: Receptors in the nerve growth factor/tumor necrosis factor receptor family are characterized by the presence of cysteine-rich motifs of ~40 amino acids in the extracellular domain. The ligands are type II transmembrane proteins with β-strands that form a jelly-roll β-sandwich. The receptors recognize soluble or cell-surface-bound ligands and mediate diverse cellular responses. Activation of intracellular signals is mediated at least in part by the association of proteins with a RING finger motif or a death domain to the cytoplasmic domains of the receptors. In addition to cell-membrane-bound receptors soluble forms have been described for most of the receptors. Activation of intracellular signals not only occurs through ligand binding to the receptors but cross-linking of at least some members of the ligand family can regulate cell functions. *J. Leukoc. Biol.* 60: 1–7; 1996.

Key Words: host defense · differentiation · cell proliferation · apoptosis

INTRODUCTION

Members of the nerve growth factor/tumor necrosis factor (NGF/TNF) receptor family are characterized by the presence of one to six cysteine-rich motifs of approximately 40 amino acids in the extracellular domain. The cysteine-rich regions provide the motif for binding to shared structures in the ligands [1, 2]. The receptor family now includes the low-affinity nerve growth factor receptor (NGFR) [3], TNFR1 (or TNFR55) [4, 5], TNFR2 (or TNFR75) [6], the TNF receptor-related protein (TNFRrp) [7], which is a Lymphotoxin (LT) β-specific receptor [8], CD40 [9], the Hodgkin's antigen CD30 [10], the T cell antigen CD27 [11], Fas/APO-1 [12, 13], OX-40 [14], and 4-1BB/ILA [15, 16]. Shope fibroma virus, cowpox virus, myxoma viruses, and vaccinia viruses contain genes that are probably acquired from the host cellular genome that encode soluble TNF receptors. The proteins are secreted from virus-infected cells, bind TNF and LT, and inhibit their biological activity [17]. The TNF ligand family includes TNF, LTα (also referred to as TNF-β), LTβ [18], the CD40 ligand gp39 [19], CD70, the CD27 ligand [20], Fas ligand [21], 4-1BB ligand [22], and OX-40 ligand [23]. The TNF ligand superfamily members, with the exception of LTα, are type II membrane glycoproteins with homology to TNF in the extracellular domain.

TNF and Fas regulate function of a broad spectrum of cell types and are implicated in diverse aspects of host defense responses and the pathogenesis of different diseases. Other members of the family, such as CD27, CD30, CD40, OX-40, and ILA/4-1BB appear to be involved primarily with the regulation of immune responses. This review will summarize structure of receptors and ligands, signal transduction, and major biological functions.

STRUCTURE OF RECEPTORS AND LIGANDS

The structure of TNFR1 extracellular domain has been determined on the basis of crystallization in complex with TNF-β [24] as well as in the absence of ligand [25]. The TNFR/TNF-β complex consists of three receptor molecules that are symmetrically bound to one TNF-β trimer. The receptor is an elongate molecule with four disulfide-rich domains in a nearly linear array and binds in the groove between two adjacent TNF-β subunits. The unliganded domains can form dimers of two distinct types [25]. Antiparallel associations occur through an interface that overlaps the TNF binding site. This form of association would separate the cytoplasmic domains and could inhibit signaling in the absence of TNF. Parallel dimers are also observed in which the dimer interface is well separated from the TNF binding site. Associations among TNF-bound parallel dimers could cause receptor clustering.

In addition to cell-membrane-bound receptors within this family soluble forms have been described for the low-affinity NGFR [26], TNFR1, and TNFR2 [27, 28], Fas [29], CD27 [30], CD30 [31], CD40 [32], and 4-1BB [33] (**Table 1**). Soluble TNFR appear to be generated by proteolytic cleavage of the membrane-associated forms because for each of these receptors only a single mRNA species has been detected. This is in contrast to the soluble form of the Fas molecule, which originates from an RNA splice variant [29]. The soluble form of Fas was also biologically active and inhibited apoptosis induced by an agonistic antibody. A mRNA splice variant of murine 4-1BB lacking the coding region for the transmembrane domain was detected in different tissues [33]. However, a

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Abbreviations: TNF, tumor necrosis factor; TNR-R, TNF receptor; NGF, nerve growth factor; LT, lymphotoxin; TRAF, TNF receptor-associated factor; SLE, systemic lupus erythematosus; NF-κB, nuclear factor-κB; Ig, immunoglobulin.

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TABLE 1. Soluble Forms of Receptors and Ligands

Receptor	Soluble form	Ligand	Soluble form
TNFR1	Cleavage	TNF	Cleavage
TNFR2	Cleavage	TNF, LTα	Cleavage
LTβR	ND	LTβ	ND
Fas	Alternate splice	FasL	ND
CD27	Cleavage	CD27L/CD70	ND
CD30	Cleavage	CD30L	ND
CD40	Cleavage	CD40L/gp39	Intracellular processing
OX-40	ND	OX-40L	ND
4-1BB	Alternate splice	4-BBL	ND
NGFR	Cleavage		

ND, not demonstrated.

similar mRNA encoding a soluble form of the human homologue ILA was not detected in an analysis of a broad spectrum of cell types. Recombinant forms of the soluble receptors that contain the entire extracellular regions or parts thereof have been important tools in characterizing the biological functions of the TNF-R and are under investigation as therapeutic agents in sepsis, arthritis, and other conditions [34].

The ligands of the TNF family are type II transmembrane proteins. The structures of TNF and LTα have been determined by X-ray crystallography [35–37]. The monomers represent eight β-strands that form a jelly-roll β-sandwich motif. Threefold related subunits form a trimer stabilized primarily by hydrophobic interactions. TNF has three sites at the interface between the subunits that can interact with the receptor. Based on modeling studies, the structures of the other ligands appear to be similar to TNF.

SIGNAL TRANSDUCTION THROUGH RECEPTORS

Activation of the TNF receptors is triggered by the aggregation of cytoplasmic domains that occurs when the extracellular domains of two or three receptors bind to trimeric TNF or LTα.

Several downstream signaling events that are activated by TNF and other ligands of the cytokine family had been characterized [38] but signaling molecules that mediate the initial interaction with the ligand-occupied receptor have only recently begun to be identified. The method used in most of these studies for the isolation of molecules that interact with the intracellular domains of the receptors was the yeast two hybrid system.

A region of 76 amino acids was identified by mutational analysis to be required for signal transduction by the TNFR2. When this region was used in affinity purification and in the yeast two hybrid system, two novel proteins, termed TNF receptor-associated factors (TRAF), were isolated. TRAF1 showed no significant sequence similarity to previously known molecules. TRAF2 contained an N-terminal RING finger sequence motif that may form zinc binding structures mediating protein-DNA or possibly protein-protein interactions. TRAF1 and TRAF2 contain a highly homologous region of 230 amino acids, called TRAF domain. This region appears to mediate heterodimer formation. Because TRAF1 showed only weak direct binding to TNF-R2, it has been suggested that TRAF2 interaction with TNF-R2 allows association of TRAF1 [39]. A protein that also contains a C-terminal TRAF domain and an N-terminal RING finger motif was identified on the basis of its interaction with the cytoplasmic domain of CD40 [40]. This molecule, termed TRAF3, also binds to an Epstein-Barr virus-encoded protein and is probably involved in signaling events leading to Epstein-Barr virus-induced B cell transformation [41]. TRAF3 self-associates but does not dimerize with TRAF1 or TRAF2 [42]. There also appears to be selectivity and specificity in the interaction of the TRAFs with the different members of the TNF receptor family. TRAF1–3 do not bind to Fas or TNFR1, TRAF1 does not interact with CD40, and TRAF3 does not bind to TNFR2 via the TRAF domain in the yeast two hybrid system [42] but it co-immunoprecipitates with TNFR2 [41]. Furthermore, with respect to downstream signaling events, there are differences. TRAF2 but not TRAF1 or TRAF3 mediates nuclear factor-κB (NF-κB) activation by TNFR2 and CD40. NF-κB activation is dependent on the presence of the RING finger motif [42].

CD40 activation on B cells inhibits programmed cell death. A zinc finger protein, A20, is induced by the Epstein-Barr virus LMP-1 gene product and inhibits B-cell apoptosis. CD40 activation induces A20 by inducible binding of NF-κB complexes to the A20 promoter [43].

The intracellular domain of Fas contains a sequence, termed death domain, that is required for the induction of apoptosis. This sequence motif is also present in TNFR1, CD40, and NGFR. Three proteins that are unrelated to the TRAF family and bind to death domains have been identified: TNFR1-associated death domain protein, TRADD, which mediates NF-κB activation and apoptosis by TNFR1 [44]. FADD and RIP bind to the death domain in Fas and, on overexpression, induce apoptosis. All three proteins contain death domains that are similar to those in the TNFR1 and in Fas and mediate interactions with the receptors.

Fas also contains a 15-amino acid C-terminal motif that functions as a negative regulatory domain that can suppress Fas-generated signals leading to apoptosis. A protein tyrosine phosphatase, termed FAP-1, interacts with this motif and the high levels of its expression correlates with the resistance to Fas-mediated cytotoxicity [45].

SIGNAL TRANSDUCTION THROUGH MEMBRANE-ASSOCIATED LIGANDS

Signal transduction occurs not only through the receptors but recent evidence suggests that cross-linking of the membrane-associated CD40L, OX-40L, and ILA/4-1BB ligand can trigger intracellular signals and regulate cell functions. The quality of a signal varies with target cell or

TABLE 2. Consequences of Genetic Defects in Receptors or Ligands

Gene	Phenotype	Reference
Spntaneous mutation		
CD40L (human)	Hyper IgM syndrome (eleated IgM) virtual absense of other isotypes	[91]
Fas (mouse)	Lymphadenopathy; autoimmune manifestations	[60]
FasL (mouse)	Similar phenotype as in Fas mutation	[47]
Knock out		
TNFR1	Resistance to LPS-induced lethality; defect in clearing *Listeria monocytogenes* infection	[55, 56]
TNFR2	Resistance to TNF-induced death and tissue necrosis	[57]
CD40	Defect in T cell dependent antibody production and isotype switching; absence of IgE	[81]
CD40L	Similar phenotype as CD40 knock out	[82]
LTα	Abnormal development of peripheral lymphoid organs	[59]

activation state. The ligand and receptor can induce qualitatively opposing effects on the same cellular response.

CD40L is expressed on activated but not on resting T lymphocytes. Much higher levels of CD40L are expressed on $CD4^+$ compared with $CD8^+$ cells. CD40 expressed on transfected cells enhanced anti-CD3-induced proliferation of $CD4^+$ cells but had only marginal effects on $CD8^+$ cells [46].

OX-40L is expressed on activated T and B cells. Cross-linking of OX-40L enhanced proliferation of B cells, immunoglobulin heavy chain mRNA levels, and immunoglobulin secretion. Cross-linking of OX-40 ligand also altered the levels of the transcription factor BSAP, thus providing direct evidence for signal transduction through this ligand [47].

Antibodies to ILA/4-1BB co-stimulate lymphocyte proliferation. However, fusion proteins containing the extracellular part of ILA/4-1BB inhibit T cell proliferation and induce cell death [48]. These effects are observed only when the fusion proteins are fixed but not in solution, suggesting that cross-linking of the ligand is required for the induction of the cellular response. This example also illustrates that the same receptor-ligand pair can induce cell functions in both receptor as well as ligand-expressing cells and that the cellular responses are qualitatively distinct, representing increased proliferation and the induction of cell death, respectively. This pattern of signaling allows a novel form of communication during cell-cell interactions. The significance of this mechanism for example in the interaction of antigen-presenting cells and lymphocytes has not yet been fully explored.

BIOLOGICAL FUNCTIONS

Biological functions of receptors and ligands in this family have extensively been reviewed elsewhere [49–53]. Here we will briefly summarize the major roles of the receptors by focusing on genetic evidence in characterizing function. Initial functional characterization for the receptors was performed with ligands, soluble receptors, and antibodies. More recently, fusion proteins that contain the extracellular part of a receptor and the constant domain of immunoglobulin G have been used in different in vitro and in vivo models. Several spontaneous mutations in the receptor genes and deletions by homologous recombination have been described (**Table 2**).

TNF and LT

TNF can induce a broad spectrum of biological effects such as cell death, gene induction, antiviral activity, and cytokine production. The TNF ligand family now includes TNF, LTα, and LTβ. In addition to cell-membrane-bound ligands within this family, soluble forms are known to occur naturally for TNF, which is synthesized as a 26-kDa precursor protein. This is processed to a secreted 17-kDa mature form by a unique Zn^{2+} endopeptidase, also termed TNF convertase [54]. The cell surface form of LTα is assembled during biosynthesis as a heteromeric complex with LTβ, a type II transmembrane protein [18]. Secreted LTα is a homotrimer that binds to distinct TNF receptors of 60 and 80 kDa. However, these receptors do not recognize the major cell surface LTα-LTβ complex. A receptor specific for human LT-β was identified, which suggests that cell surface LT may have functions that are distinct from those of secreted LTα [8]. Gene targeting of TNFR1 confirmed its role in the lethality in response to low doses of lipopolysaccharide after sensitization with D-galactosamine but the toxicity of high doses of lipopolysaccharide was unaffected. TNFR1 mutant mice were severely impaired in their ability to clear infection with the facultative intracellular bacterium *Listeria monocytogenes* [55, 56]. TNFR2-deficient mice show normal T cell development and activity but have increased resistance to TNF-induced death and a decrease in TNF-induced tissue necrosis [57]. Studies on fibroblasts from TNFR1-deficient mice suggested that this receptor controls adhesion to leukocyte cell lines as well as ICAM-1, VCAM-1, CD44, and MHC class I up-regulation, secretion of other cytokines, cell proliferation, and NF-κB activation. Stimulation through TNFR2, in TNFR1-deficient fibroblasts, did not have any effect in these functions [58].

Mice deficient in LTα by gene targeting have no morphologically detectable lymph nodes or Peyer's patches, although development of the thymus appears normal. Within the white pulp of the spleen there is failure of normal segregation of B and T cells. Spleen and peripheral

blood contain $CD4^+/CD8^-$ and $CD4^-/CD8^+$ T cells in a normal ratio, and both T cells subsets have an apparently normal lytic function. Lymphocytes positive for immunoglobulin M are present in increased numbers in both the spleen and peripheral blood. Thus, LTα appears essential in the normal development of peripheral lymphoid organs [59].

Fas/APO-1

Fas, also termed APO-1, was discovered as a cell membrane receptor, which, upon activation by specific antibody, triggered cell death by apoptosis. The lymphoproliferation (*lpr*) mutation in the MRL strain of mice is caused by the insertion of a transposable element in the Fas gene. The insertion causes a decrease in Fas mRNA expression and the Fas protein is not expressed on resting or activated lymphocytes from MRL lpr/lpr mice. These findings suggest that Fas plays a role in both thymic selection and T cell survival in the periphery and that the accelerated autoimmunity in MRL lpr/lpr mice results from a defect in both of these pathways [60].

Recombinant Fas ligand induced apoptosis in Fas-expressing target cells. Fas ligand is expressed in activated splenocytes and thymocytes, consistent with its involvement in T cell-mediated cytotoxicity and in several nonlymphoid tissues [21]. The MRL mouse strain with generalized lymphadenopathy (*gld*) develops similar autoimmune manifestations as the *lpr* strain. The gld mutation is a point mutation in the Fas ligand that abolishes binding to the receptor [61].

A potential association of an impairment in the induction of apoptosis and human systemic lupus erythematosus (SLE) has been suggested. Peripheral blood mononuclear cells from SLE patients produced increased levels of a soluble form of Fas. This receptor competes for binding of Fas ligand and protects cells from apoptosis [29]. Possible consequences could be a persistence of autoreactive lymphocytes or the release of undegraded DNA from necrotic cells, which could stimulate the formation of anti-DNA antibodies.

CD27

CD27 is a transmembrane homodimer with subunits of 50–55 kDa expressed only on lymphoid cells, including the majority of peripheral T cells, a subset of B cells, NK cells, and CD3 bright thymocytes. During lymphocyte activation the expression of CD27 increases and a soluble 28- to 32-kDa form of CD27 (sCD27). One mRNA encodes both the transmembrane receptor and sCD27. The transmembrane form gives rise to sCD27 most likely via a proteolytic event [62]. sCD27 has been detected in body fluids from healthy individuals [30, 52].

CD27 co-stimulates proliferation and enhances cytokine synthesis of T cells that are activated by mitogens, antigens, or antibodies to CD2, CD3, or CD28 [63, 64]. This receptor is also involved in the PWM-driven T cell-dependent IgG synthesis [64].

CD27 is expressed on most but not all peripheral blood $CD4^+$ T cells. The small fraction of $CD4^+$ T cells with a $CD27^-$ phenotype exclusively resides within the $CD45RA^-$ $CD45RO^+$ subset. $CD27^-$ cells are functionally differentiated cells that have lost CD27 expression as a result of persistent antigenic stimulation. $CD27^+$ and $CD27^-$ cells do not differ notably in the expression of CD70 (CD27 ligand) [65, 66]. CD27 is also expressed on a subpopulation of human B lymphocytes and positively correlated with membrane immunoglobulin (Ig) A but negatively correlated with membrane IgM/membrane IgD positivity. CD27 on B cells can be induced selectively by the combination of *Staphylococcus aureus* plus interleukin-2. After in vitro stimulation, $CD27^+$ but not $CD27^-$ B cells secrete large amounts of both IgM and IgG. CD27 may thus be a marker that discriminates naive from primed B lymphocytes [67].

These functions of CD27 on lymphocytes were confirmed with CD27 ligand. Cloned CD27 ligand co-stimulated T cell proliferation and enhanced the generation of cytolytic T cells [68], cytokine production, induction of activation antigens, and proliferation of unprimed $CD45RA^+$, and to a lesser extent, of primed $CD45RO^+$ peripheral blood T cells [69]. CD27L is identical to the previously identified activation antigen CD70. CD70 expression in vivo is confined to activated B and T lymphocytes [70]. On T cells, CD70 was expressed almost equally on both activated CD4 and CD8 cells. On subsets of CD4 T cells, however, CD70 expression was induced preferentially on the CD45RO T cell population after activation, whereas its expression was not seen on CD45RA T cells [71].

CD30

CD30 was originally described as a cell-surface antigen on primary and cultured Hodgkin's and Reed-Sternberg cells [53]. CD30 is normally expressed by a subset (15–20%) of $CD45RO^+$ T cells after activation by a variety of T cell stimuli. $CD30^+$ T cells are preferentially regulated by IL-12, and the effects of IL-12 on T cell IFN-γ production are mediated largely through its effects on the $CD30^+$ subset. $CD30^+$ T cells also secreted higher levels of IL-5 than activated $CD30^-$ T cells. In contrast $CD30^-$ T cells produced significantly higher levels of IL-2 than $CD30^+$ T cells. $CD30^+/CD4^+$ T cells exhibit significantly greater helper activity for B cell Ig production than $CD30^-/CD4^+$ T cells. Thus, $CD30^+$ T cells are the major interferon-γ- and interleukin-5-producing T cells, and exhibit potent helper activity for Ig production [72, 73].

Soluble CD30 is released by T cell clones and tumor cells. High serum levels of sCD30 were observed in atopy, SLE, and after infection with measles virus or human immunodeficiency virus [74].

Recombinant human CD30 ligand enhanced Ig secretion of Epstein-Barrvirus-transformed B-cell lines and increased proliferation of some tumor cells, whereas in others it induced cytolytic cell death [75]. CD30 is expressed

constitutively on the human T cell line ACH-2, which is chronically infected with HIV-1. Cross-linking CD30 results in HIV expression, which is associated with NF-κB activation and enhanced HIV transcription [76].

CD40

CD40 is expressed on B lymphocytes, thymic epithelial cells, activated monocytes, dendritic cells, hematopoietic progenitor cells, epithelial cells, and carcinomas. Cross-linking of CD40 with immobilized anti-CD40 or cells expressing CD40L induces high levels of B cell proliferation and addition of IL-4 or IL-13 allows the generation of factor-dependent long-term normal human B cell lines and the secretion of IgE following isotype switching [77].

CD40 ligand (CD40L), a 39-kDa glycoprotein, is transiently expressed on activated T cells, mostly $CD4^+$ but also some $CD8^+$ as well as basophils and mast cells. Soluble CD40L is an 18-kDa protein that is generated by intracellular processing [78, 79]. Individuals with X-linked hyper-IgM syndrome fail to express functional CD40L and, as a consequence, are incapable of mounting protective antibody responses to opportunistic bacterial infections [80].

In CD40 and RAG-2 knock-out mice where all mature lymphocytes were derived from the CD40-deficient embryonic stem cells, T and B cell number and phenotype were normal. However, $CD40^{-/-}$ chimeras completely failed to mount an antigen-specific antibody response or to develop germinal centers following immunization with a T cell-dependent antigen but responded normally to T cell-independent antigens. The $CD40^{-/-}$ animals had an absence of IgE and a severe decrease of IgG1 and IgG2a [81]. Similar results were obtained with mice deficient in CD40L expression [82]. These results support the essential role of CD40- CD40L interactions for T cell-dependent antibody responses and in isotype switching and show that Ig class switching to isotypes other than IgE can occur in vivo in the absence of CD40L. CD40 also mediates various functional effects on other cell types [83].

A role in the pathogenesis of collagen-induced arthritis has been suggested by studies where administration of gp39 antibodies reduced disease severity and decreased the titers of antibodies to type II collagen [67].

OX-40

OX-40 expression appears to be restricted to activated T cells [84] where it acts as a costimulatory receptor. Cloning of the ACT35 lymphocyte activation antigen revealed that it corresponds to human OX-40 [85].

Human OX-40 ligand, gp34, was previously known to be expressed by T cell lymphotropic virus 1-infected cells. Recombinant OX-40 ligand expressed in COS cells costimulates phorbol myristate acetate, phytohemagglutinin, and anti-CD3-induced $CD4^+$ T cell proliferation [23].

Expression of OX-40L was detected on activated T cells, with higher levels found on $CD4^+$ than $CD8^+$ cells [86]. OX-40 ligand was also expressed on a subset of peritoneal B cells and LPS-activated splenic B cells [84]. Cell-bound recombinant ligands co-stimulate T cell proliferation and cytokine production, in particular IL-4 secretion [86].

4-1BB/ILA

4-1BB was initially identified as a gene that is inducibly expressed by murine T lymphocytes [15, 87]. Cross-linking of 4-1BB enhanced anti-CD3-induced T cell proliferation [88] as well as the proliferation of anti-u-primed splenic B cells [89]. ILA, the human homologue of 4-1BB, was also discovered as a gene that is expressed in activated T cells [16]. In addition to T cells, ILA is also expressed on B lymphocytes, monocytes, epithelial cells, and chondrocytes [90]. Expression in all of these cell types is activation-dependent. Antibodies to ILA co-stimulated anti-CD3-induced proliferation of human T lymphocytes. However, when anti-CD3-stimulated T cells were cultured in the presence ILA-IgG fusion protein in solid-phase, it inhibited proliferation and induced apoptosis [48]. This was not observed with soluble fusion protein, suggesting the possibility that 4-1BB ligand is capable of providing an anti-proliferative or death-inducing signal to the cells.

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Tumor Necrosis Factor Receptor Superfamily

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ABBREVIATIONS:

LTα	lymphotoxin alpha
sTNF-R1	soluble extracellular domain of the type I tumor necrosis factor receptor
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha
TNF-R	tumor necrosis factor receptor
TNF-R1	type I (55 kDa) tumor necrosis factor receptor
TNF-R2	type II (75 kDa) tumor necrosis factor receptor

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Tumor necrosis factor (TNF) is a powerful cytokine which is involved in the immune and pro-inflammatory response. The TNF receptors (TNF-R1 and TNF-R2) are the sole mediators of TNF signaling. The receptors consist of a disulfide rich domain which recognizes TNF, a transmembrane helix, and a cytoplasmic domain. Signaling occurs when a TNF trimer binds two or three receptors in an extracellular complex which permits aggregation and activation of the cytoplasmic domains. The complex is then endocytosed where it dissociates at low pH. We have now determined the structure of the soluble extracellular domain of TNF-R1 in two crystal forms at pH 3.7 in addition to our earlier report of one form at pH 7.5. One low pH form diffracts to 1.85 Å and the entire polypeptide sequence has now been traced for this protein. The C-terminal 20 residues of the protein which were disordered in all previous structures show a different topology and disulfide connectivity to that seen in the remainder of the structure. In all crystal forms, the uncomplexed soluble extracellular domain of the type I TNF-R (sTNF-R1) exists as a dimer. At low pH the dimer buries a large amount of solvent accessible surface (2,900 Å^2), over 800 Å^2 greater than the area buried by TNF complexation. This dimer at low pH is different than both dimers observed in our previous pH 7.5 structure of unliganded sTNF-R1. We suggest that the low pH dimer forms during endocytosis and as the dimer completely buries the TNF interaction surface, the dimer would break up the receptor TNF complex. We have identified two distinct structural modules in sTNF-R1, a type A and a type B module. We suggest that these modules are the unit of structural conservation rather than the 6 cysteine subdomain. Although the orientation of these modules with respect to each other is sensitive to crystal packing, complexation, and pH, the modules themselves are structurally well conserved between and within the known sTNF-R1 structures. This modular approach will allow us to build accurate models for all members of the TNF-R superfamily. © *1996 Wiley-Liss, Inc.*

INTRODUCTION

The effects of TNF are signaled through two receptors, type I (55 kDa) and type II (75 kDa). Despite the name, the anti-cancer activity of TNF has been of little therapeutic benefit, due to the high systemic toxicity of the factor. However, iso-

lated limb perfusion of high doses of TNF has been reported to provide medical benefit. The realization that excessive TNF signaling may be responsible for the deleterious effects of diseases such as arthritis, inflammatory bowel diseases, bacterial meningitis, and septic shock has shifted interest to controlling TNF signaling.

The receptor is composed of an N-terminal cysteine rich extracellular domain, connected via a transmembrane helix to a C-terminal intracellular domain [1]. Residues 12–171 of the intact type I receptor (sTNF-R1), effectively the entire extracellular domain, is found free in serum and urine, generated by a shedding event at the membrane [2]. sTNF-R1 regulates signaling by sequestering free TNF. TNF binds to the extracellular domains of two or three receptor molecules promoting the aggregation and activation of the intracellular domains. The activated intracellular domains bind other proteins triggering the signal cascade. The complex is then endocytosed with concomitant fall in pH, and dissociates. This model agrees with the 2.85 Å crystal structure of LTα complexed to sTNF-R1 [3]. LTα is found as a trimer and the trimer has a β-barrel shape. Three sTNF-R1 molecules are bound parallel to the trimer axis along the outside surface of the barrel. One sTNF-R1 molecule is bound at each of the three LTα/LTα interfaces. The receptor is an elongated molecule composed of repeating disulfide subdomains which result in a ladder-like structure of disulfide bonds. The interactions with LTα are confined to two separate regions of the receptor: a lower region and an upper region. No interactions were found between the receptors.

Intracellular domains expressed free from the extracellular and transmembrane domains and in the absence of TNF will aggregate and signal [4] along the same pathway as conventional TNF-induced signaling. Intact receptors are tightly regulated and are silent in the absence of TNF. One interpretation of these results is that in some way the extracellular domains prevent "false" signaling in the absence of TNF. We have previously reported the structure of unliganded sTNF-R1 at pH 7.5 [5]; this showed two distinct dimers. We suggested that either or both of these dimers may form on the cell surface in the absence of TNF preventing signaling. We also pointed out that one dimer may have been able to bind TNF promoting large cell surface aggregates which may play a role in complex internalization or may concentrate the signal within the cell. However, we also noted that such aggregates were not seen in the crystalline TNFα.sTNF-R1 complex [3].

The TNF receptors belong to the low affinity nerve growth factor receptor (NGF-R) superfamily which includes FAS, CD40, OX40, CD27 receptor, and several viral proteins. The defining characteristic of this family is an extracellular domain comprised of cysteine repeats. These proteins are structurally distinct from the cysteine knot class of proteins such as human chorionic gonadotropin [6]. Aside from the presence of a cysteine rich sequence there is little homology across the superfamily at the extracellular level; at the intracellular level there is no homology at all across the family. The lack of homology at the intracellular level is unsurprising given the diverse range of activities signaled by the receptors. The specificity of recognition of the appropriate cytokine is thus crucially important for cell viability. Inspection of the sequences of the extracellular domains of these receptors suggests a repeating pattern of cysteines and disulfides. The repeating unit suggested was a 6 cysteine, 3 disulfide subdomain shown in Figure 1. The first structural view of this subdomain came from the structure of the LTα sTNF-R1 complex. This was followed by a structure of the unliganded receptor [5]. These structures showed the that sTNF-R1 contained two classical subdomains and a third distorted subdomain. The fourth subdomain of the structure was partially disordered and the four C-terminal cysteine residues were not visualized. This was important as it seemed from the sequence that the four cysteines could not adopt the classical subdomain pattern. Many of the other members of the superfamily similarly fail to fit the 3 cysteine, 6 disulfide subdomain.

We have now determined a complete high res-

FIGURE 1

A ribbon representation of the structure of sTNF-R1 with disulfide bonds shown.

FIGURE 2

The low pH dimer is formed by an anti-parallel combination of two monomers. Interactions occur along the length of protein involving all four subdomains.

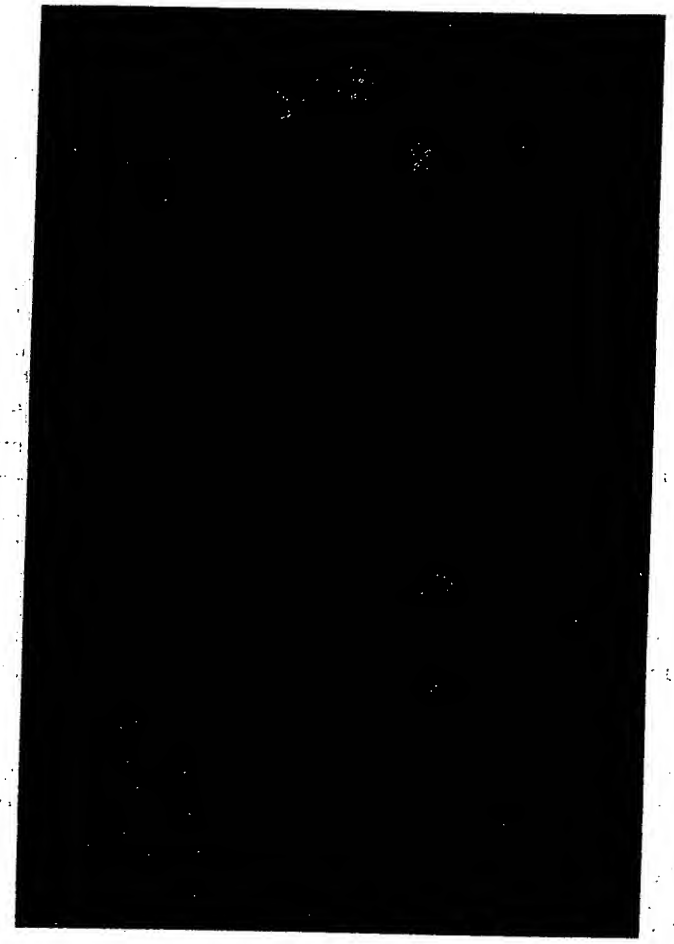

FIGURE 1

FIGURE 2

olution structure of sTNF-R1. This structure shows that indeed the disulfide bond pattern in the fourth subdomain does not fit the classical subdomain arrangement. We have identified two new repeating units of structure which we believe are conserved rather than the 3 cysteine, 6 disulfide subdomain.

RESULTS AND DISCUSSION

Structure of the Monomer

The monomer in the $P2_12_12_1$ crystal form is shown in Figure 1. The receptor is highly elongated. The fold of the three N-terminal subdomains (15–52, 55–96, 98–137) and the N-terminal half of the fourth subdomain (139–150) remain essentially unchanged from earlier descriptions [3,5]. Briefly, each subdomain has two S-shaped modules. The A (N-terminal) module is composed of 12–17 residues which form 3 short strands with a single disulfide bond. The B (C-terminal) module is composed of 21–24 residues which again form 3 short strands linked with 2 disulfides. The C-terminus of the fourth subdomain (153–171), which was disordered in earlier studies, is clearly different from the C-termini of the other three subdomains. The disulfide pattern is Cys153–Cys166 and Cys156–Cys162 and the lobe contains 15 amino acids. The sequence "encapsulation" of the second disulfide by the first disulfide leads to an extremely distorted structure.

Low pH Dimer

In both crystal structures, sTNF-R1 is found as a dimer (the low pH dimer; Fig. 2). The low pH dimer is different than the dimers found at pH 7.5. The low pH dimer is formed by an anti-parallel (head to tail) arrangement of the monomers. The two monomers completely overlap, such that the length of the dimer is 90 Å, almost identical to that for a single monomer. The dimer interaction buries a surface area of 2,880 Å^2. In the $P6_122$ crystal form which diffracts to 1.87 Å the same dimer is found but in a different crystal packing environment. However, the C-terminal 38 residues are disordered in this structure and it is not possible to fully quantify the dimer interactions for this crystal form. The low pH dimer is held together by extensive interactions burying 1,480 Å^2 hydrophobic surface area and 1,402 Å^2 hydrophilic surface area. The interactions are found along the entire length of the protein. The low pH dimer buries almost 50% more surface area than the LTα sTNF-R1 complex. Residues 14–17, 21, 60, 69–72, 77–79, 104–107, 110–114, 144, 146–147, 151–155, and 160–161 are involved in or are buried by formation of the low pH dimer; this includes virtually all of the residues involved in LTα recognition [3,7]. This dimer would obviously be incapable of binding TNF as its interaction surface is masked. We suggest that during endocytosis of the TNF TNF-R1 complex, the receptors form the low pH dimer disrupting the complex. As yet no direct evidence exists to support a dimeric receptor species in solution. A large entropy penalty was suggested to explain the lower than expected dimerization affinity for human chorionic gonadotropin in solution [8] despite the removal of nearly 4,000 Å^2 from solvent by dimerization. Attached to a membrane as an intact receptor, the entropy penalty for dimerization would be significantly lowered for TNF-R1.

pH 7.5 Dimers

We have previously reported in detail the interactions which form the two dimers observed at pH 7.5 [4,7,9]. Briefly, the parallel dimers are formed by a head to tail arrangement of the sTNF-R1 monomers shown in Figure 3A. Residues 17–19, 31–37, 47–49, 54, 64, 90, 91, 126, 127, 130, 133, 136–138, and 145 are involved in dimer formation. This recognition surface for the parallel dimer is primarily confined to the first subdomain, with some involvement of the third subdomain and very small contributions from the second and fourth subdomains. The dimer buries 2,143 Å^2 of surface area (1,071 Å^2 per monomer); 1,064 Å^2 is

FIGURE 3
A: The parallel dimer found at pH 7.5, this dimer is capable of binding TNF. B: The anti-parallel dimer found at pH 7.5, this dimer is unable to bind TNF.

FIGURE 4
Superposition of the $P2_12_12_1$ structure and that complexed to LTα. The large change in the position of the N-terminus is visible.

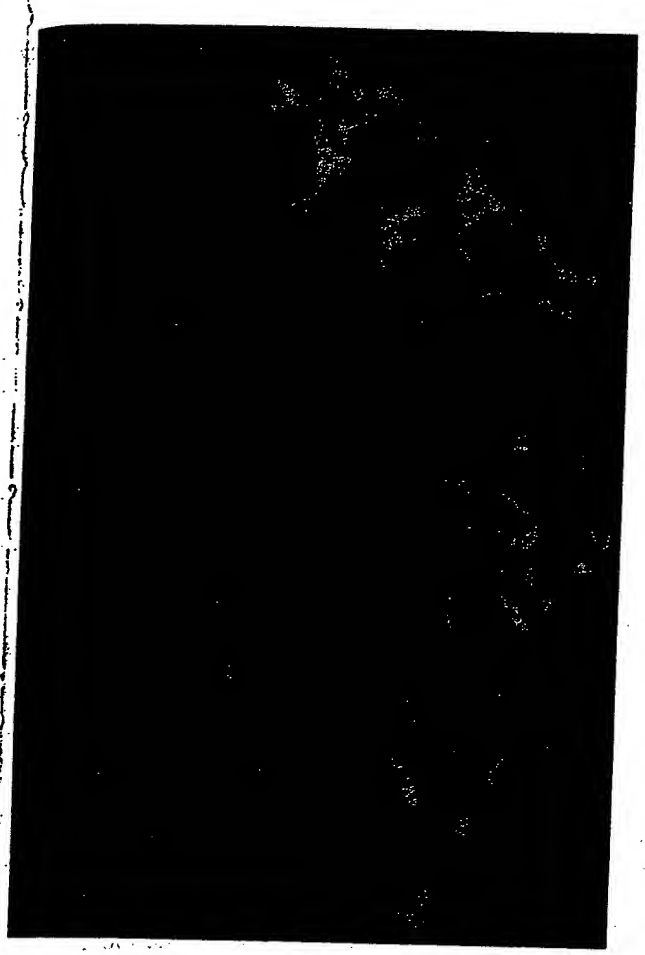

FIGURE 3A

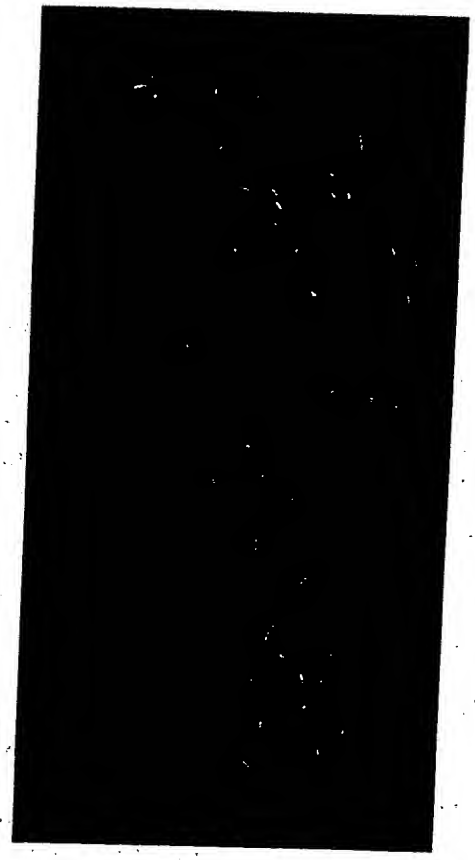

FIGURE 4

FIGURE 3B

polar and 1,079 Å^2 is apolar. A simple modeling experiment shows that it is possible to dock a TNF trimer at each monomer in the parallel dimer without introducing steric conflict. It seems possible, therefore, that the parallel dimer could survive in the presence of TNF. In this dimer, the cytoplasmic domains would be close together, below the membrane surface.

The anti-parallel dimer is formed by a head to tail arrangement of monomers and is shown in Figure 3B. Residues 24, 40, 41, 53, 59, 60, 69–79, and 93 are all involved in anti-parallel dimer formation. The contacts are principally confined to subdomain 2, although subdomain 1 is weakly involved. In all, the interaction buries 1,475 Å^2 of surface area (737 Å^2 per monomer), which can be divided into 878 Å^2 polar and 597 Å^2 apolar buried surface area. Unlike the parallel dimer, the anti-parallel dimer cannot bind TNF and would be disrupted by exposure to TNF. In the anti-parallel dimer the long axis lies in the plane of the membrane, separating the cytoplasmic domains by over 100 Å.

Structural Conservation Within sTNF-R1

The existing paradigm is that the repeating unit is a 6 cysteine containing subdomain, with sequence Cys1 x_{10-15} Cys2 x_2 Cys3 x_2 Cys4 x_{8-11} Cys5 x_{7-8} Cys6. The disulfide pattern is Cys1–Cys2, Cys3–Cys5, Cys4–Cys6. This unit is exemplified by subdomains two and three of sTNF-R1, which superimpose reasonably well. However, subdomain one, although having the same disulfide bonding pattern as subdomains two and three, does not superimpose. The reason for this is that there are no intervening residues between Cys2 and Cys3 in subdomain one, whereas there are two residues in subdomains two and three. This results in different orientation of the A module with respect to the B module in subdomain one. In subdomain four although there are 6 cysteine residues, the disulfide spacing is inconsistent with the subdomain type structure. Our structure shows the fourth subdomain has both a different disulfide connectivity and structure. Taking sTNF-R1 as a whole the repeating unit is only found in half the structure, although large parts of structure are similar. We believe that the 6 cysteine subdomain is not the unit of structural conservation but merely a frequently occurring piece of structure. We suggest that there are two distinct (but probably related) repeating units, an A type module and a B type module. The 6 cysteine subdomain is simply a linear AB combination. The modules are linked by zero to two intervening amino acids. These points of linkage serve as hinges to accommodate conformation changes in gross structure. In subdomain one where there is little hinging movement it is notable that there are no intervening residues.

There are four A1 (number denotes number of disulfide bonds) type modules in sTNF-R1, at residues 15–29, 55–70, 98–114, and 139–150. Each module has strand turn strand turn strand topology, with sequence Cys1 x_2 Gly x Tyr x^a x^b_{4-9} Cys2. The loop x^b_{3-8} is structurally variable, however, the remainder of the module is structurally well conserved.

There are three B2 modules with sequence Cys3 x_2 Cys4 $x_{3-6}x^c_5$ Cys5 x^d_{4-5} D T x Cys6 at residues 30–52, 73–96, and 117–137. The first strand of four amino acids runs from the Cys3 to Cys4; a non-structurally conserved turn of between three and five amino acids follows. The loop x^d_4 D T x superimposes well for the subdomains two and three both contain four amino acids. In subdomain one there are five amino acids in this region, including two prolines. This region does not superimpose well on the other two, however, the remaining residues do. Inspection of superfamily sequences suggests that the first type B module (residues 30–52) in sTNF-R1 appears to be unusual and that the x^d_4 loop is by far the most frequently occurring loop [10].

Differences Among the Various Structures of sTNF-R1

We now have six monomers of sTNF-R1, two in $P2_12_12_1$ (pH 3.7), one in $P6_122$ (pH 3.7), two in $P4_12_12$ (pH 7.5), and one in the LTα sTNF-R1 complex. Overall we observe large differences in the structures of the protein dependent on the pH, whether it is complexed to TNF, and the precise packing environment. These differences are equivalent to movement in the N-terminus of nearly 20 Å (Fig. 4). The cause of these differences is hinging motion at the module connections. The structures of the underlying modules are remarkably well conserved among the various crystal structures. We view this as confirmatory evidence for the modules as the structural unit of conservation.

TNF-R Superfamily

We face two particular problems in extending our analysis to other members of the family: the

first is an "excess" of cysteines in a given sequence and the second is a paucity of cysteines. Excess cysteine regions are found in several members of the TNF-R superfamily. We believe the regions with an excess of cysteines are A2 modules; i.e., they have the same structure as type A1 modules, but have an additional, sequence encapsulated, disulfide bond. Banner et al. [3] suggested such an internal disulfide for sTNF-R2; we concur and suggest that this is not an exception but a common structural feature. The number of amino acids in these excess cysteine regions and the conservation of the aromatic residue are consistent with an A type module structure. We believe this is the only model consistent with structural conservation for "excess" cysteine regions.

Cysteine poor regions are also found in many members of the TNF-R superfamily, often concurrently with excess cysteine regions. We believe these cysteine poor regions are B1 modules, i.e., they have the same structure as a B2 modules, but miss the Cys3–Cys5 disulfide bond. Our evidence for this is that the length of the module and the conservation pattern, the C x_4 D T x C found at the C-terminus of the module, is consistent with the type B module. Cys 5 is commonly mutated to Gly or Ala and Cys 3 to a His or Trp (although several combinations are observed). Again this appears to be the only model consistent with structural conservation.

We favor our modular approach for various reasons. First the structures of the type A and B modules are structurally well conserved within sTNF-R1, in response to pH and complexation, even when the connections between them are not. Finally, this method does appear to produce a consistent structure-based alignment of disparate TNF-R superfamily members (Naismith and Sprang, manuscript in preparation).

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Modularity in the TNF-receptor family

James H. Naismith and Stephen R. Sprang

Tumour necrosis factor (TNF) receptor family members regulate processes that range from cell proliferation to programmed cell death. The extracellular, ligand-binding domains of these proteins consist of small, cysteine-rich subdomains, first observed in the three-dimensional structures of the type I TNF receptor. A structure-based alignment of TNFR family members indicates that the extracellular domains are constructed primarily of two small polypeptide modules. These modules play distinctive structural roles in the architecture of the domains. Analogues of at least one of these modules can be found in the domains of other receptors and extracellular proteins. Variations in their sequence and order of assembly are expected to account for differences in shape, flexibility and ligand specificity.

MEMBERS OF THE tumour necrosis factor receptor (TNFR) family initiate signals that can lead to programmed cell death or promote cell survival and proliferation[1,2]. The family includes the low-affinity nerve growth factor receptor, type I (p55) and type II TNF (p75) receptors (TNFR-1 TNFR-2, respectively), FAS (CD95), CD40, CD27 and their relatives[3,4] together with the newly discovered mediators of apoptosis DR3/Wsl-1/Apo-3/TRAMP[5–8] and DR4 (Ref. 9). Therapeutic interest has centred on controlling the signalling of cytokines, particularly TNF, which has been implicated in a growing list of autoimmune diseases[10]. Certain virally produced TNFR homologues can suppress the host immune response by sequestering TNF[11]. Entry of Herpes Simplex virus into cells is mediated by a member of the TNFR family[12].

Although diverse in primary structure, the extracellular domains of all TNFR family members consist of cysteine-rich subdomains[3] that are thought to adopt generally similar tertiary folds. Nevertheless, it is the unique structural features of individual family members that allow them to recognize their ligands with specificity and, in most cases, exclusivity.

The mechanisms by which these molecules achieve specificity cannot be understood without knowledge of their three-dimensional structures. In the absence of crystallographic or NMR structural data, molecular modelling can provide clues to function. To be useful, models must reflect the correct alignment between consensus sequences and reliable tertiary structural templates. Here, we show that the extracellular domains of TNFR family members can be parsed into repeats of two basic types of modular unit, each of which is distinguished by a characteristic consensus sequence and tertiary fold.

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Cysteine-rich repeats

The primary structures of the extracellular domains of TNFR-1 and TNFR-2 (Refs 13–15) appear to comprise repeats of a 6-cysteine, 3-disulphide subdomain. The first view of these subdomains was provided by X-ray crystallographic studies of sTNFR-1 (soluble extracellular domain of TNFR-1) determined both in complex with lymphotoxin α[16] (LT) and in the unliganded state[17,18]. Three of the four subdomains of sTNFR-1 (residues 15–52, 55–96 and 98–137) are topologically equivalent, exhibiting the disulphide connectivity illustrated in Fig. 1. In three crystal structures, including that of the complex with LT, the C-terminal 20 residues, including four cysteines, cannot be visualized. In crystals of sTNFR-1 grown at low pH and high salt, the fourth subdomain is well ordered. Its C-terminal half adopts a disulphide connectivity and topology different from that of the other three subdomains[18] (Fig. 1). This observation supports the suggestion by Bazan[3] that the basic building block of receptor structure is not the 6-cysteine, 3-disulphide unit, but rather a pair of smaller modules. These two modules can be combined in several ways to produce the diverse group of molecules listed in Table I.

The modules

We suggest the extracellular domain of TNFR-1 contains three topologically distinct types of modules, which we term A1, B2 and C2 (Fig. 1). The letter denotes the type of module and the numeral the number of disulphide bridges within the module. The 'classical' 6-cysteine, 3-disulphide subdomains of TNFR-1, for example residues 98–137, are in fact an A1 → B2 pair, whereas the fourth subdomain (residues 139–166) is an A1 → C2 pair (see Fig. 1a).

The four A1 modules in sTNFR-1 contain a structurally conserved core, as do the three B2 modules (Fig. 1b). Indeed, the structural conservation among the A1 and B2 modules is striking[18], despite the differences in sequence and length among these modules within sTNFR-1. In the four distinct crystal packing environments in which the structure of sTNFR-1 has been determined, the structure of these modules is insensitive to protein contacts. We suggest that in the TNFR superfamily there are occurrences of A-modules with two disulphide linkages (A2 modules) and B-modules with only one disulphide (B1 modules). We predict that the structures of A2 modules are similar to A1 modules, and likewise, B1 modules are structurally similar to B2 modules. The C2 module appears unique to TNFR-1. Although most TNFR homologues comprise a series of AB repeats, this is not always the case. For example, Fas begins with a B2 module, and both 41BB and OX40 contain tandem repeats of A-modules (Table I).

A-modules

A1 modules are composed of 12–17 residues and contain one disulphide (Table II). They are defined by the consensus sequence Cys1-x_2-Gly-x-Tyr(or Phe)-x-x_{4-9}-Cys2. In the foregoing notation, x_n is the number of intervening amino acids. The A1 module is C-shaped and consists of three short β-strands linked by turns (Fig. 2a). The disulphide bond (Cys1–Cys2) connects strand 1 to strand 3. The loop connecting the second and third strands is highly variable, and can contain between four and nine residues. The remainder of the A1 module is structurally conserved despite the absence of sequence conservation. We suggest that it is the length, rather than the composition

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of the variable loop that determines its structure. The distribution of loop lengths in TNFR-1 is typical of the family. Therefore, the structures of many of the A1 modules listed in Table I could be modelled upon those in the crystal structure of sTNFR-1.

Many TNFR-1 homologues, for example TNFR-2, contain modules with the A1 consensus sequence, but with an additional pair of cysteine residues (Table II). Banner *et al.*, suggested that these are joined to form a second internal disulphide bond within the domain[16]. A2 modules have sequence Cys1-x_2-Gly-x-Tyr(Phe)-Cys3-x_{4-9}-Cys4-x_2-Cys2. Our model predicts an additional disulphide between strands 2 and 3 (Cys3–Cys4; Fig. 2a) with the remainder of the structure unchanged. The number of amino acids and the conservation of the aromatic and glycine residues are consistent with the A-module structure.

As with the A1 modules, the second loop (connecting Cys3–Cys4) is variable in length (Table II). Using an A1 module of sTNFR-1 (residues 15–29) as a template and substituting Thr21 and Ile26 with cysteine residues, we can model an A2 module (Fig. 2b). Formation of the additional disulphide bond requires only modest (<1.0 Å) rearrangements of the backbone. Interestingly, A2 modules, but not A1 modules, show a strong preference for a bulky hydrophobic residue (Tyr, Trp, Phe, Met) preceding the conserved aromatic residue (Table II). A phenylalanine present at this position (Phe143) in the fourth A1 motif in sTNFR-1 stacks against the module's disulphide bond, presumably stabilizing it. This may be related to the fact an A2 module is typically not followed by the more common and possibly more stable B2 module (Table I).

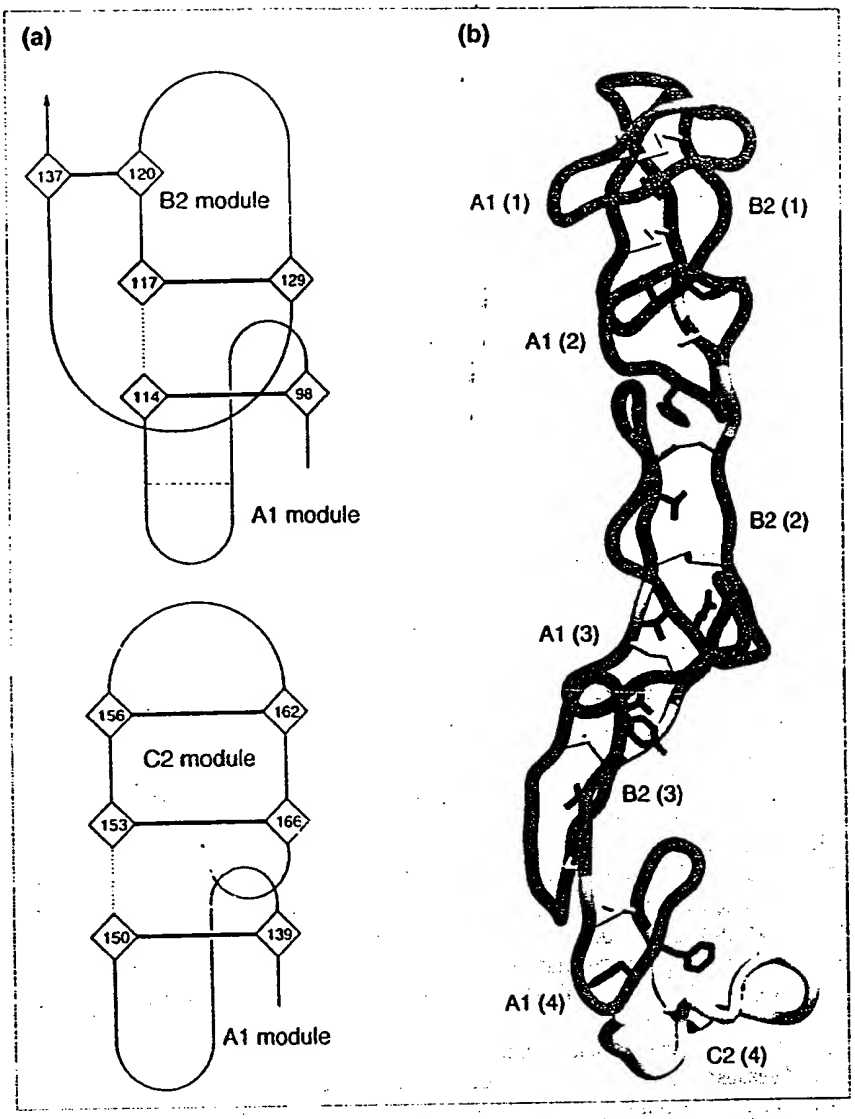


Figure 1

Architecture of the soluble extracellular domain of tumour necrosis factor receptor 1 (sTNFR-1). **(a)** Schematic representation of the 6-cysteine subdomain, which had been proposed to be the basic repeating structural unit of the extracellular domain of the TNFR family (top) and the fourth subdomain of sTNFR-1, which has a different structure (bottom). The position of the proposed disulphide bond between strand 2 and 3 in A2 modules is shown with a dashed line. **(b)** The complete structure of the extracellular domain of TNFR-1 (Ref. 18). A-modules are shown in magenta and B-modules in blue. Disulphide bridges (yellow) and the sidechains that pack between them are shown.

B-modules

B2 modules consist of 21–24 residues and comprise three antiparallel strands that adopt an S-shaped fold reminiscent of three-quarters of a paper clip (Fig. 2b). B2 modules contain the consensus sequence Cys1-x_2-Cys2-x_{3-6}-x_5-Cys3-Thr-x_{2-5}-Asn-Thr-Val-Cys4, with disulphide cross-links Cys1–Cys3 and Cys2–Cys4 (Table III). The stabilizing role of the Asn-Thr-Val motif has already been discussed[16] and is evident in Fig. 2b. The conserved threonine following Cys3 hydrogen bonds to a backbone amide group of the third strand, stabilizing the module. One example of this is found in sTNFR-1 (Thr89) but has been omitted from Fig. 2b for clarity.

The loop connecting strand 1 to strand 2 varies in length (3–6 amino acids) in sTNFR-1. Many of B2 modules that occur in other members of the family can be modelled, assuming that length and not sequence determines the structure. A glycine residue is favoured in this loop. The loop connecting strand 2 to strand 3 is much less variable and is predominantly three residues long, although other lengths are found (Table III). The remainder of the structure is identical despite the lack of sequence conservation.

B1 modules lack the Cys1–Cys3 disulphide bond. However, because they are similar in length and consensus sequence to B2 modules (Table III), it is likely that they adopt the same three-dimensional structure. The residue corresponding to Cys1 in a B2 module is commonly replaced by histidine or tryptophan, and Cys3 by glycine or alanine (although several combinations are observed) (Table III). Figure 2b shows a histidine/glycine combination from CD40. The histidine can either form a weak hydrogen bond with or pack against strand 2.

A- and B-modules in other proteins

The extracellular, ligand-binding domains of the low-density lipoprotein (LDL) families of receptors contain

Table I. Modular composition of receptors

Receptor (residues)	Modules[a]	Acc. No. (Ref.)[b]
TNFR-1 (15–166)	×0 ×2 ×2 ×1 ×2 ×1 ×2	P19438
41BB (28–158)	×2 ×2 ×1 ×3 ×2 ×2	Q07011
CD27 (27–120)	×0 ×2 ×2 ×1	P26842
CD30 (29–500)	×0 ×3 ×2 ×1 ×2 U[c] E1 ×2 ×2 ×2	P28908
CD40 (26–186)	×0 ×2 ×2 ×1 ×2 ×2 ×2	P25942
CrmB (30–168)	×0 ×2 ×2 ×1 ×2 ×2	(26)
CrmC (37–185)	×0 ×2 ×2 ×1 ×2 ×2 D2	(27)
DR3 (9–152)	×0 ×2 ×2 ×1 ×2 ×2 ×2 F2	(28)
DR4 (109–206)	×2 ×2 ×1 ×2	(29)
FAS[f] (59–165)	×2 ×2 ×1 ×2	P25445
HVEM (42–179)	×0 ×2 ×2 ×1 ×2 B0 ×2	(30)
NGF-R (32–188)	×0 ×2 ×2 ×1 ×2 ×2 ×2	P08138
OSTE (41–185)	×2 ×2 ×1 ×2 ×2 ×2	(31)
OX40 (31–166)	×0 ×2 ×2 ×1 ×2 ×2	P43489
TNFR-2 (40–200)	×0 ×2 ×2 ×1 ×2 ×2 ×2	P20333
TNFRC (43–210)	×0 ×2 ×2 ×1 ×2 ×2 ×2	P36941
VC22[g] (32–168)	×0 ×2 ×2 ×1 ×2 ×2	P21071
VT2Myx[g] (28–174)	×0 ×2 ×2 ×1 ×2 ×2	P29825
VT2Sfv[g] (28–174)	×0 ×2 ×2 ×1 ×2 ×2	P25943

Key: A1 module; A2 module; B1 module; B2 module; C2 module; Outliers

[a]Loop structures are shown as white boxes with the number of residues indicated (×n). [b]Where the SWISS-PROT accession number is not known, the reference is given. [c]83 intervening amino acids that are not cysteine rich. [d]The disulphide connectivity in DR3 is ambiguous. [e]A2 in DR3 does not fit conventional A2 pattern. [f]The mouse sequence has an N-terminal A1 module before the B2 module. [g]These sequences contain cysteines C-terminal to the last module, which may be responsible for dimerization of the receptors.

Table II. Examples of A1 and A2 modules[a]

Receptor	Amino acid sequence (single letter code)[b]												
	Strand 1 to strand 3 disulphide												
A1 modules													
TNFR-1 (15–29)	C	P	Q	G	K	Y	I	H	–	x5	–	I	C
TNFR-1 (55–70)	C	E	S	G	S	F	T	–	–	x8	–	–	C
TNFR-1 (98–114)	C	R	K	N	Q	Y	R	H	Y	x5	F	Q	C
TNFR-1 (139–150)	C	H	A	G	F	F	L	R	E	–	N	E	C
TNFR-2 (40–53)	C	R	L	R	E	Y	Y	–	–	x6	–	–	C
CD27 (27–39)	C	P	E	R	H	Y	W	–	–	x5	–	–	C
DR3[c] (9–23)	C	D	C	A	G	D	F	H	–	x5	–	F	C
Typical A1 sequence	C	x	x	G	x	ϕ[d]	x	–	–	x4–9	–	–	C
	Strand 1 to strand 3 disulphide												
A2 modules													
41BB (88–102)	C	T	P	G	F	H	C	x4	–	C	S	M	C
CD30 (108–125)	C	R	P	G	M	F	C	x7	–	C	A	R	C
CrmC (116–135)	C	L	P	G	W	F	C	x9	–	C	R	D	C
DR3[c] (93–114)	C	K	P	G	W	F	x2	C	x4	C	x7	–	C
TNRC (126–142)	C	Q	P	G	M	F	C	x6	–	C	T	H	C
Typical A2 sequence	C	x	p	G	π[e]	θ[f]	C	x4–7		C	x	x	C
							Strand 2 to strand 3 disulphide						

[a]SWISS-PROT accession numbers and references are given in Table I. [b]Alignment is such that residues in the same column are structurally conserved. Where xn appears, this is a loop structure, n denotes the number of residues. Amino acids in red are well conserved (or conservatively substituted). [c]The disulphide bond may be from Cys11 to Cys23 in DR3, and it does not fit the standard A2 model. [d]ϕ represents Tyr or Phe. [e]π is the favoured bulky hydrophobic residue. [f]θ represents Tyr, Phe or His.

disulphide-rich complement-type and EGF-type repeats. The disulphide connectivity and tertiary structures of these repeats clearly distinguish them from the subdomains of the TNFR family. Nevertheless, superposition of the solution structures of LDL-complement repeats 1 (rLB1) (Ref. 19) and rLB2 (Ref. 20), with the crystal structure of TNFR-1 demonstrates that the N-terminus of rLB2 contains an A-module (Fig. 2c).

The x_7 loop in rLB2 is flexible, but most conformers adopt a structure that is similar to that in the corresponding loop in sTNFR-1. rLB1 has an A1 module at its N-terminus, with a loop of different length (5 residues). The most persuasive argument for the similarity between the rLB N-termini and A1 modules is the presence and role of the conserved aromatic residue that, in both cases, stacks against a disulphide bond.

Ward *et al.* have suggested that the disulphide-rich subdomains in other extracellular proteins (insulin receptors, epidermal growth factor receptors) bear structural similarity to sTNFR repeats[21]. Indeed residues 241–253 of the human

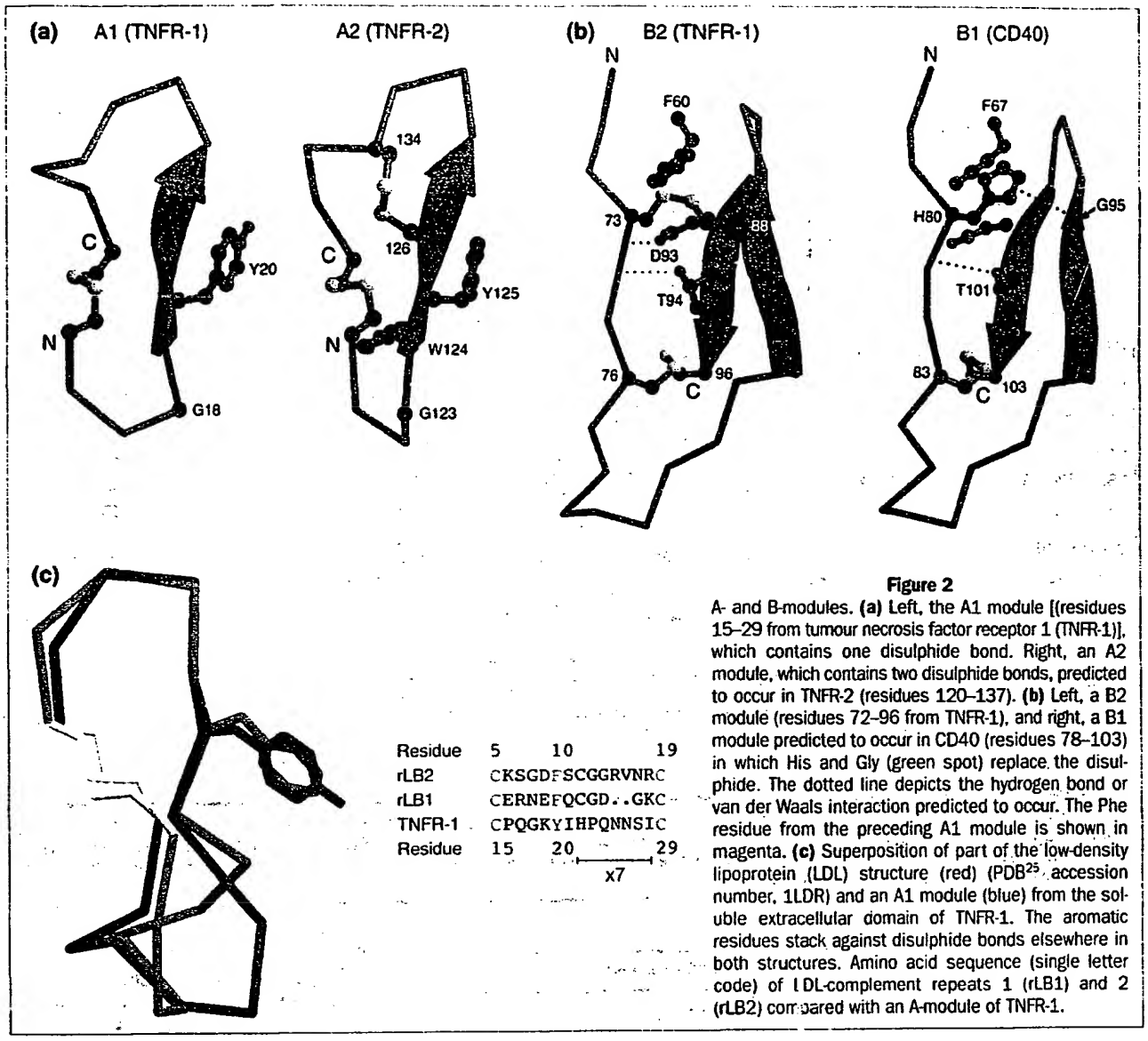


Figure 2

A- and B-modules. **(a)** Left, the A1 module [(residues 15–29 from tumour necrosis factor receptor 1 (TNFR-1)], which contains one disulphide bond. Right, an A2 module, which contains two disulphide bonds, predicted to occur in TNFR-2 (residues 120–137). **(b)** Left, a B2 module (residues 72–96 from TNFR-1), and right, a B1 module predicted to occur in CD40 (residues 78–103) in which His and Gly (green spot) replace the disulphide. The dotted line depicts the hydrogen bond or van der Waals interaction predicted to occur. The Phe residue from the preceding A1 module is shown in magenta. **(c)** Superposition of part of the low-density lipoprotein (LDL) structure (red) (PDB[25] accession number, 1LDR) and an A1 module (blue) from the soluble extracellular domain of TNFR-1. The aromatic residues stack against disulphide bonds elsewhere in both structures. Amino acid sequence (single letter code) of LDL-complement repeats 1 (rLB1) and 2 (rLB2) compared with an A-module of TNFR-1.

insulin receptor appear to have an A1 sequence. However, the predicted disulphide connectivity for these receptors would appear in some cases to preclude A- or B-type module structures.

B-modules are similar to the T-knot folds that have been observed in many small three-stranded, disulphide-rich domains[22,23]. They differ, however, in that the disulphides sandwich the threonine residue in the Asn-Thr-Val motif (Fig. 2b) rather than directly stack against each other[24]. The modules are too small and share too little sequence identity to state whether they have diverged from a common ancestor.

Outliers

Not all of the cysteine-rich subdomains found within TNFR-1 homologues are readily recognized as A- or B-modules. The C-terminus of the Cowpox CrmC-like proteins we have denoted as D2 modules, residues 233–243 in CD30 as an E1 module and residues 155–171 of DR3 as an F2 module (Table I). We have no crystallographic template for the structures of these modules. Residues 141–162 of HVEM are reminiscent of a B1 module (Gly-Thr-x_3-Asp-Thr-Leu-Cys at the C-terminus), but the predicted first cysteine (Cys144) is actually a serine. We denote this a B0 module, but cannot predict the effect of the substitution on the module structure.

Connecting A- and B-modules

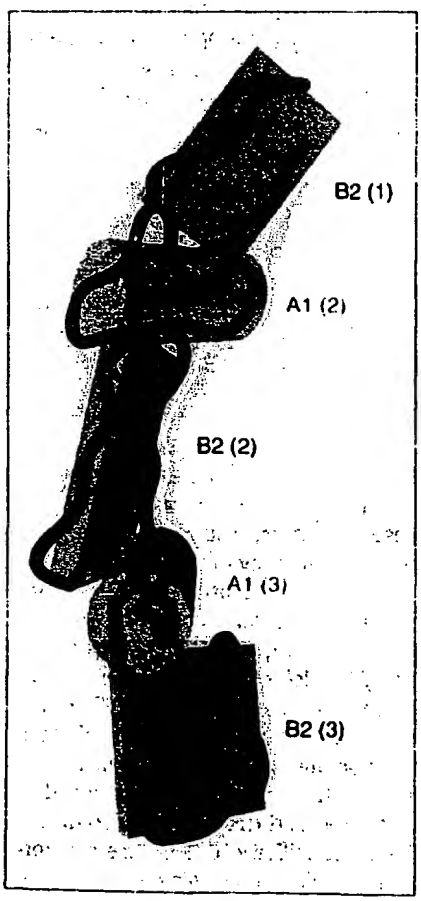
The structure of sTNFR-1 is similar to a spiral of overlapping hinges (Fig. 3) in which the B-modules correspond to the plates and the A-modules to the bolts about which they pivot. Gly97 in sTNFR-1, which sits almost exactly in the middle of the structure, has already been identified as highly flexible. It permits the structure either side to make considerable rigid body movements (equivalent to 10 Å at the N- and C-terminii)[18]. The connecting regions, which separate the modules, contain none, one and two intervening amino acids. The modules in TNFR-1 are joined such that the successive disulphide bridges describe a smooth superhelix. We predict that the conformations of the linker regions, regardless of their length, will preserve this structural feature.

The interfaces between A- and B-modules are often tightly packed cores bounded by the disulphide bridges from the flanking modules (Fig. 1b, 3) irrespective of whether one considers an A → B pair or a B → A pair [for example residues 98–137 or residues 117–150, respectively in sTNFR-1 (Fig. 1b)]. The precise nature of the interface differs depending on the combination. In the A → B

Table III. Examples of B2 and B1 modules[a]

Receptors (residues)	Amino acid sequence (single letter code)[b]
B2 modules	Strand 1 to strand 3 disulphide
TNFR-1 (30–52)	C T K C x4 Y L Y N D C P x4 – – D F D C
TNFR-1 (73–96)	C S K C x6 V E I S S C T V D R D F V C
TNFR-1 (117–137)	C S L C x3 T V H L S C Q E K Q N I V C
41BB (28–45)	C S N C – – x2 G T F C D N N R N Q I C
41BB (65–86)	C R Q C x4 R T R K E C S S T S N A E C
CD30 (45–65)	C Y R C x4 F P T Q Q C P – x2 – P T D C
CD30 (84–106)	C V T C x5 V E K T P C A W N S S R V C
CrmB (86–108)	C – x3 C x4 V E T R S C N T T H N R I C
FAS (59–82)	C – x3 C x4 R K A R D C T x4 – – E P D C
FAS (146–165)	C T K C x2 G I I K E C T L T S N T K C
Typical B2 sequence	C x x C x4 x x x x x C T x x x N T V C
	Strand 1 to strand 2 disulphide
B1 modules	Strand 1 to strand 3 disulphide
41BB (136–158)	W T N C x5 S V L V N G T K E R D V V C
CD30 (128–149)	H S V C x4 I V K F P G T A Q K N T V S
CD40 (80–103)	H K Y C x6 R V Q Q K G T S E T D F I C
CrmB (130–150)	Q T K C x3 Y G V S G H T P T G D V V C
CrmC (138–157)	K R K C x2 G Y F G G I D E L G N P L S
TNFR-2 (182–200)	H Q I C – x2 V A I P G N A S R D A V C
Typical B1 sequence	H x x C x4 x x x x x G T x x x N T V C
	Strand 1 to strand 2 hydrogen bond

[a]SWISS-PROT accession numbers and references are given in Table I. [b]Alignment is such that residues in the same column are structurally conserved. Where xn appears, this is a loop structure, n denotes the number of residues. Amino acids in red are well conserved (or conservatively substituted).



interface, residues from the A-module, including the conserved aromatic, pass between disulphide linkages. The B → A interface is formed by residues from the B-module sandwiched between the pair of disulphide linkages. In this manner, a ladder of disulphide bridges is formed, spaced by approximately 10 Å. In cases where the A → B connection is less than two residues, the disulphides from adjacent modules stack directly upon each other[21]. The interface between the first A1 module (residues 15–29) and the second B2 module (residues 30–55) in TNFR-1 is an example (Fig. 1b).

Functional implications

We believe our approach of using A- and B-modules to build receptors (Table I) will lead to more accurate and hence useful molecular models for many members of the TNFR superfamily. These models will greatly aid in probing ligand–receptor recognition. Ligands for members of the receptor superfamily can have completely different structures, making it difficult to draw general conclusions for the entire family of ligand–receptor complexes. Only the complex of LT with TNFR-1 has been structurally characterized[16]. In this complex, the receptor uses three modules (residues 50–114) to bind LT. The variable loop regions of the A- (especially) and B-modules carry most of the LT-binding residues. This may be a general feature of the interaction of ligand with receptor. The core of the A- and B-modules serve as a structural scaffold that can be decorated with short regions of variable length and amino acid sequence. These variable regions confer specificity for the appropriate ligand.

The combination of structural conservation and sequence variability allows each member of this large receptor superfamily to recognize its cognate ligand with exquisite specificity while using a small array of topologically distinct units. A model of CD40 (based on A- and B-modules) and its interaction with its CD40 ligand, shows these variable loops to be important in this complex also (J. Singh, pers. commun.). The ability to adapt to maximize recognition may be enhanced by the flexibility of the connections between the A- and B-modules. The relative orientation of the A- and B-modules is probably crucial for recognition/specificity. We noted that in sTNFR-1 that these movements allowed the receptor to bind to itself (in three distinct dimers) or to LT with a high degree of complementarity[18].

Figure 3
A hinge and bolt model for soluble extracellular domain of tumour necrosis factor receptor 1 (sTNFR-1). The schematic shows residues 30–137, A-modules are shown as magenta cylinders and B-modules as blue plates. The TNF-binding site is contained mainly within the projecting loops of the A1 modules. The conserved aromatic residues are shown intercalated between the disulphide bridges (yellow).

Conclusion

Members of the TNFR family, in common with other, apparently unrelated extracellular cysteine-rich proteins, are constructed of modular units. As with TNFR-1, many large and perhaps unrelated extracellular disulphide-rich proteins are likely to be constructed with a limited repertoire of topologically distinct modules. The modules can accommodate a great deal of sequence diversity while conserving their structure allowing large families of highly ligand-specific receptors to evolve.

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Mechanistic parallels between DNA replication, recombination and transcription

Thomas Kodadek

The multiprotein complexes that mediate replication, transcription and homologous recombination in eukaryotic cells face many of the same molecular challenges. These include the recognition of DNA sites embedded in large chromatinized genomes, the denaturation of duplex DNA, and partial dissociation and reassociation at different stages of the catalytic cycle. Therefore, it is not surprising that several steps in the respective catalytic cycles are strikingly similar at the DNA level and may proceed by similar mechanisms. Some of these relationships are reviewed here. It is argued that speculation based on such 'crosspathway' comparisons may be a valuable paradigm for the design of new experiments.

EUKARYOTIC TRANSCRIPTION, replication, and homologous recombination are mediated by very large 'protein machines'[1] comprising many interacting polypeptides. Unraveling the detailed mechanisms of action of these protein machines and elucidating in detail how regulators influence their activity is a daunting problem that occupies hundreds of laboratories around the world. Investigators look routinely to the corresponding prokaryotic systems for guidance because these are better characterized biochemically. A less common paradigm is for analyses of eukaryotic transcription, for example, to be predicated on knowledge gained from studies of recombination or some other aspect of DNA metabolism.

To a large extent, the fields of replication, transcription and recombination are separate, with their own meetings and a copious literature that makes it difficult for scientists (including this author) working in one area to remain current in the others. However, a consideration of each pathway at the DNA level (Fig. 1) reveals striking similarities. Each requires: (1) recognition of a specific initiation site; (2) unwinding of the initiation site and assembly of a primed DNA- and/or RNA-synthesizing holoenzyme; and (3) elongation of a primer, which requires movement of the protein machine away from its assembly point. Do these parallels carry over to detailed mechanism? It is suggested here that 'crosspathway' thinking could provide valuable mechanistic insight and, at the very least, suggest interesting avenues of investigation. Owing to space constraints, I will restrict my comments to homologous, rather than site-specific recombination, and RNA polymerase II (pol II)-mediated transcription, but not pol I- or pol III-mediated events. A consideration of non-recombinational DNA-repair pathways is also beyond the scope of this article.

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Finding a needle in a haystack

The binding of specific DNA sequences by a protein in the midst of the vast excess of nonspecific DNA represented by a large eukaryotic genome is a significant chemical-recognition problem[2,3]. For example, a multiprotein complex called transcription factor IID (TFIID) marks the point at which the pol II transcription complex will be assembled[4]. The human genome [~10^9 base pairs (bp)] is thought to contain perhaps 10^5 promoters for mRNA-encoding genes, meaning that there is about a 10^4-fold excess of nonspecific DNA sites over promoter sites. If we define binding selectivity as K_d(nonspecific)/K_d(specific), then a selectivity for an 'average promoter' over an 'average nonspecific site' of about 10^5-fold would be required for TFIID to saturate 90% of these promoters, assuming TFIID is limiting. An occupancy of 99% would require a 10^6-fold discrimination. Eukaryotic origins of replication are recognized by a six-protein origin recognition complex (ORC)[5,6] which plays a role in replication roughly equivalent to that of TFIID in transcription. The precise number of replication origins in a human cell is unknown, but is thought to be several thousand.

Do TFIID and the ORC have sufficient binding specificity to find core promoters and replication origins, respectively,

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REVIEW

Structure–activity studies of human tumour necrosis factors

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The mechanism by which tumour necrosis factors (TNF and lymphotoxin, also called TNFα and TNFβ respectively) exert their cytotoxic activity on many malignant cells, remains largely unknown. Furthermore, the broad array of differentiation (gene induction) and mitogenic activities towards many primary cells is still a subject of intensive investigation. TNF is an important mediator in inflammation, immune responses and infection-related phenomena and these activities contribute to the severe toxicity seen when TNF is used as an anticancer agent. The first step in the mechanism of action is the specific binding of the ligand to its receptors and dissection of the molecular mechanism involved in this interaction is the subject of this review. The reasons for the interest in this aspect are obvious: first, the development of strong antagonistic TNF analogues can be useful in dampening the potentially lethal or debilitating effects of an overproduction of the cytokine (as in septic shock or rheumatoid arthritis). Secondly, since two distinct TNF receptors exist, construction of TNF muteins that distinguish between both types may lead to derivatives of this pleiotropic agent with a more restricted biological activity pattern. Ideally, one would like to develop a TNF mutant that has retained its cytotoxic action on tumour cells without inducing the deleterious systemic toxicity. Such an optimized TNF molecule could become a potent anticancer agent.

Key words: active site/lymphotoxin/mutagenesis/structure–function/tumour necrosis factor

Primary structures

Since the cloning of human and mouse tumour necrosis factor (TNF) and lymphotoxin, some 8 years ago (Gray *et al.*, 1984, 1987; Pennica *et al.*, 1984, 1985; Fransen *et al.*, 1985; Marmenout *et al.*, 1985), the genes for rabbit (Ito *et al.*, 1986), rat (Shirai *et al.*, 1985), cat (McGraw *et al.*, 1990), dog (Fiers, 1993), porcine (Pauli *et al.*, 1989; Drews *et al.*, 1990), goat (Sprang and Eck, 1992), bovine (Niitsu and Watanabe, 1988), equine (Su *et al.*, 1991) and ovine (Young *et al.*, 1990; Green and Sargan, 1991) TNF and for rabbit (Ito *et al.*, 1986) and bovine (Niitsu and Watanabe, 1988) lymphotoxin have been isolated by different groups (Figure 1). Conservation within the TNF and lymphotoxin sequences is 71 and 61% respectively and comparison between the two human cytokines reveals an identity of 32%, when the alignment was optimized, based on the comparison of their 3-D structures (Eck *et al.*, 1992; see below). Cloning of the cDNA for the human and mouse membrane-bound T-cell antigen CD40L (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992) revealed an identity of 22% when the extracellular domain of human CD40L was aligned with the human TNF and human lymphotoxin amino acid sequences (Figure 1). The corresponding receptor for this ligand is the B-cell CD40 antigen, a member of a family of transmembrane proteins, including the low-affinity nerve growth factor (NGF) receptor and the two different TNF receptors, TNF-R55 and TNF-R75 (see below). Recently, a fourth member of the TNF family, called p33 or lymphotoxin-β, was identified and found to be 23 and 27% identical to TNF and lymphotoxin respectively (Figure 1; Browning *et al.*, 1993). p33 forms a heteromeric complex with lymphotoxin at the cell surface (see below).

Asparagine-linked glycosylation was demonstrated on murine TNF and on murine and human lymphotoxin, at positions 7, 60 and 62 respectively (Haranaka *et al.*, 1986; Porter, 1990), but not on human TNF (potential *N*-glycosylation sites can also be found in several other species). Moreover, it was shown that *O*-glycosylation on Thr7 could contribute to the heterogeneous molecular mass forms of natural human lymphotoxin preparations (Voigt *et al.*, 1992). Also CD40L is supposed to be *N*-glycoslated since its calculated M_r (29 395 Da) does not correspond to its observed M_r (32–33 000 Da) (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992). Except when indicated in this report, positions in the protein will be numbered, starting from Val1 of human TNF. Negative numbering stands for residues in the pre-sequence with Ala1 being the first residue upstream of Val1.

Tertiary structure

X-ray diffraction data on TNF (Eck and Sprang, 1989; Jones *et al.*, 1989, 1990a, 1991; Sprang and Eck, 1990, 1992) and lymphotoxin crystals (Eck *et al.*, 1992) reveal the 3-D structures of both cytokines (Figure 2a and b). Each subunit, folded into a 'jelly-roll' antiparallel β-sheet sandwich, forms a bell-shaped, rigid, trimeric molecule with two other subunits. As a result, a channel is formed along the trimer axis, lined by charged and polar residues near the top and bottom and by hydrophobic residues in the middle part. Conserved residues between TNF and lymphotoxin cluster predominantly in the inner core of the molecule, when a slightly different alignment was used also (Tavernier *et al.*, 1989). As expected, the exterior surface is composed mostly of charged and polar residues. The monomers show a striking structural homology with some viral capsid proteins such as influenza haemagglutinin and especially satellite tobacco necrosis virus (STNV) coat protein. The only cysteine bridge in each TNF monomer is localized near the top, while the N- and C-termini are located at the bottom of the structure. Dimers of trimers have been reported (Sprang and Eck, 1990), but it remains to be established whether such a higher order quaternary structure represents a biologically relevant entity rather than a crystallographic curiosity.

Since the regions of homology between CD40L or p33 and TNF/lymphotoxin fall within the β-sandwich forming sequences (Figure 1), a similar 3-D fold has been suggested for CD40L

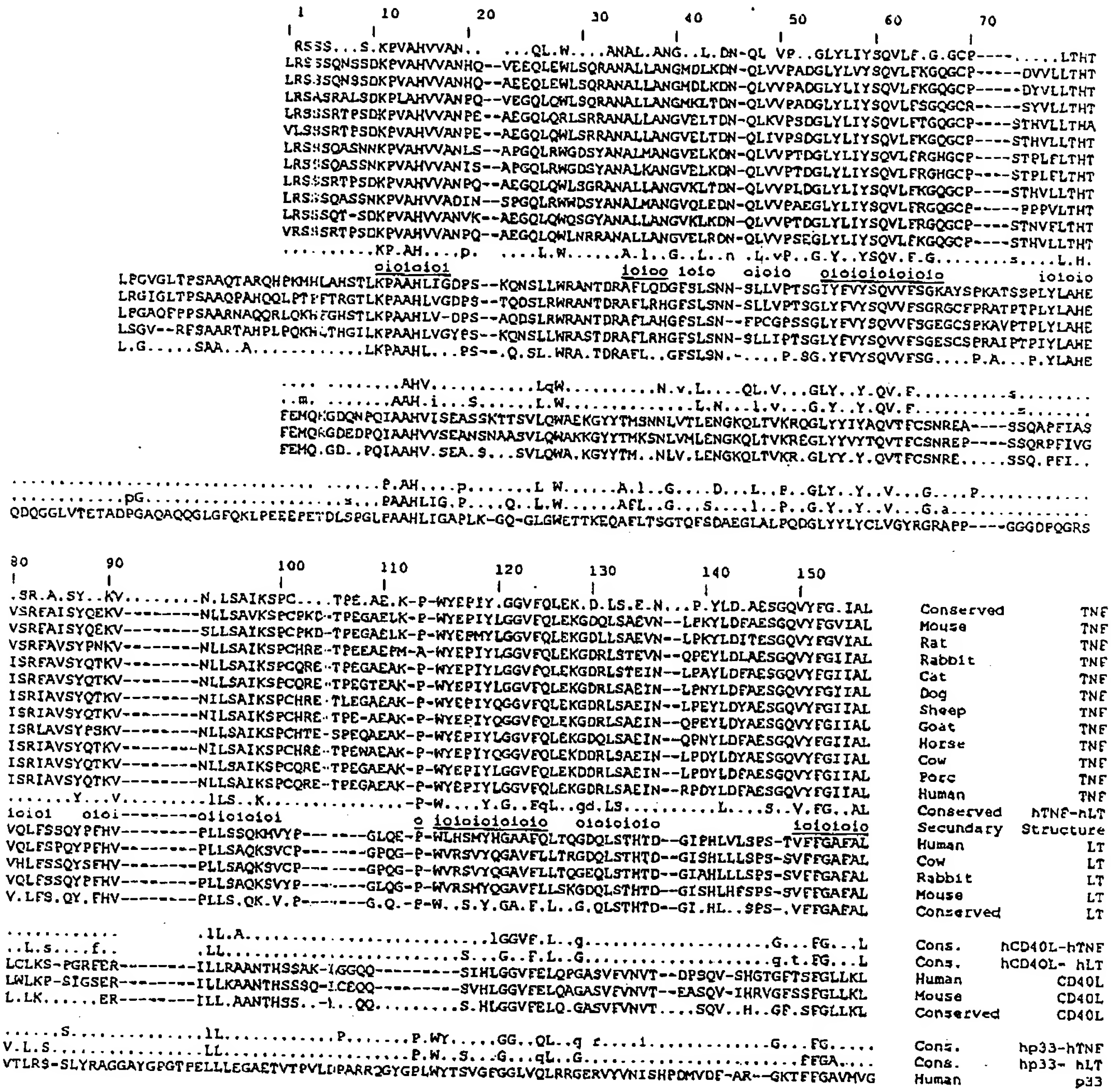


Fig. 1. Primary sequence of different mammalian TNF, lymphotoxin, CD40L and p33 species (see text for references). Conserved TNF, conserved LT and conserved CD40L stand for identical residues in the different TNF (one exception is accepted), lymphotoxin and CD40L species respectively. Conservation between human TNF and human lymphotoxin, conserved hTNF–hLT; between human CD40L and human TNF, cons. hCD40L–hTNF; between human CD40L and human lymphotoxin, cons. hCD40L–hLT; between human p33 and human TNF, cons. hp33–hTNF, between human p33 and human lymphotoxin, cons. hp33–hLT. Small letters indicate that the conservation in one of the structures is not fully conserved between the different species (for TNF, one exception is accepted). Residues involved in secondary structure of TNF and lymphotoxin (line 14) are indicated as i and o (= inside and outside orientation in the monomer β-sandwich respectively). β-Strands on the sheet facing the trimer axes are underlined (adapted from Sprang and Eck, 1992).

(Farrah and Smith, 1992) and p33 (Browning *et al.*, 1993). Hence, it can be proposed that, in analogy with the hypothesis of Bazan (1990) for the cytokine receptor family, the ligands of the TNF/NGF receptor family also share some structural (and even sequential) homology. However, although NGF is also an all-β protein, its topology is completely different from TNF and lymphotoxin (McDonald *et al.*, 1991) and the TNF/NGF ligand family could thus fall into two structural classes (Farrah and Smith, 1992).

Membrane-anchored variants

Human TNF is synthesized as a precursor protein of 233 amino acids. The prosequence has an unusual length of 76 amino acids and is very conserved in all species of the TNF family (76%), suggesting an as yet unknown but important biological role. In this prosequence, a hydrophobic stretch between residues −44 and −26 is present (Fiers *et al.*, 1991b). Since it was shown that TNF can also function as a surface-bound cytotoxin (Decker *et al.*, 1987; Liu *et al.*, 1989), this stretch could serve as a

membrane anchor of the TNF prohormone, as has been demonstrated by several groups (Kriegler *et al.*, 1988; Luettig *et al.*, 1989; Kinkhabwala *et al.*, 1990). The myristyl acylation of a couple of lysine residues, upstream of this sequence, may facilitate the membrane insertion process (Stevenson *et al.*, 1992). Pro-TNF should thus have a type II membrane glycoprotein configuration since its C-terminus is located extracellularly and this characteristic is shared with the CD40L ligand (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992).

The unusual processing of this precursor molecule is remarkable, since no release of the 17 kDa mature TNF subunit was detected in a cell-free system, supplemented with dog pancreas microsomes, but processing to TNF polypeptides of various lengths (17, 18.5 and 20 kDa) was observed in mammalian cells and *Xenopus laevis* oocytes (Müller *et al.*, 1986). It has been suggested that the TNF precursor is transported to the cell surface and then cleaved by extracellular protease activity, thereby leaving behind a 14 kDa prosequence in the cell membrane (Jue *et al.*, 1992). The difference in calculated (8660 Da) and apparent (14 kDa) M_r and the absence of glycosylation, supports the idea of other post-translational modifications of the membrane-inserted prosequence, such as myristyl acylation (Jue *et al.*, 1992). An 18.5 kDa mature subunit was also characterized in the medium upon induction of the mouse RAW264.7 cell line and it was shown that this variant started with ten additional N-terminal amino acids that rendered the molecule biologically inactive (Cseh and Beutler, 1989). In this respect, it may be mentioned that the *in vitro* translated precursor TNF is also inactive (Kriegler *et al.*, 1988), suggesting that segments of the prosequence can mask the active centre or constrain the molecule into an inactive form. Because cleavage within the region between amino acids -32 and -1 should result in the 17, 18.5 and 20 kDa forms, Klostergaard *et al.* (1992) deleted this sequence and replaced it by the tetrapeptide Ile–Asp–Leu–Glu, but unexpectedly no uncleavable forms could be found. A similar result was observed when small deletions within the −3, +5 region were introduced (Perez *et al.*, 1990). A deletion of the first 12 amino acids disrupts the cleavage process of TNF and leaves a membrane-bound, active form (Perez *et al.*, 1990).

The signal sequence of lymphotoxin (34 amino acids) is more standard and does not allow membrane anchoring. Nevertheless, membrane-bound lymphotoxin was detected (Abe *et al.*, 1991; Hiserodt *et al.*, 1992). Here, anchoring occurs by the non-covalent interaction of one lymphotoxin monomer with a membrane-bound dimer of a p33 protein (Browning *et al.*, 1991; Androlewicz *et al.*, 1992). Further studies must unravel whether this lymphotoxin–p33 heterotrimer exerts activity and, if so, how far it mimics that of lymphotoxin. Certainly, this capacity of the lymphotoxin monomer to combine with other molecules is, in view of its structure–function relationship, an intriguing phenomenon that could shed new light on the mechanism of action of lymphotoxin.

Physicochemical characteristics

A summary of the biochemical and biophysical properties of TNF and lymphotoxin is given in Table I. Many attempts have been made to determine the mol. wt of native TNF and lymphotoxin by gel filtration, but aspecific interactions with the different column matrices (Yoshimura *et al.*, 1980; Petersen *et al.*, 1989; Schoenfeld *et al.*, 1991) were at the source of confusing conclusions. Moreover, conflicting reports have been published, concerning the amount of monomeric TNF in solution (Narhi and Arakawa, 1987; Smith and Baglioni, 1987; Schoenfeld *et al.*, 1991; Corti *et al.*, 1992). However, methods such as ultracentrifugation and sedimentation, cross-linking, small angle light scattering and 2-D gel electrophoresis showed that TNF under all physiological circumstances is in a trimer form (Table I).

On the whole, TNF is relatively resistant to organic solvents and acidic conditions as compared with lymphotoxin (Aggarwal, 1990; Porter, 1990). The side chain of Lys98 in TNF forms an ionic bond with the carboxylate group of Glu116 from the same subunit and this results in a stabilizing ring of ion pairs around the 3-fold axis near the top of the molecule. Since Glu116 is replaced in lymphotoxin by a histidine residue, the lack of charge compensation may account for the acid lability of the molecule (Eck *et al.*, 1992). In contrast, lymphotoxin is more resistant to protease activity. Indeed, several highly accessible protease recognition sites are exposed near the bottom of the TNF molecule, where the putative active site is located (see below), while in lymphotoxin, these sequences map near the less sensitive N-terminal part or the upper region of the molecule (Jones *et al.*, 1991).

It should be noted that, although unglycosylated lymphotoxin has the same bioactivity as the glycosylated natural product, the presence of the carbohydrate group renders the molecule more hydrophilic (Hains and Aggarwal, 1989) and makes it more accessible for iodination (Stauber and Aggarwal, 1989).

The modification of TNF with reagents specific for the imidazol moiety of histidine (Yamamoto *et al.*, 1987, 1989) or for the free amino groups of lysine and the N-terminal valine (Klostergaard *et al.*, 1992; Utsugi *et al.*, 1991, 1992), resulted in a decrease of cytotoxicity, correlating with the degree of TNF modification. This led in the former case to the identification of His15 as a possible candidate for participation in the active site (see below). On the other hand, acylation of some amino groups can lead to an increase in hydrophobicity without loss of TNF activity and such a modified TNF molecule can integrate more easily into lipid vesicles. It has been claimed that lipid-bound TNF possesses unchanged *in vitro* tumor cytotoxicity and immunomodulatory effects, while some toxic side-effects *in vivo* would be reduced (Debs *et al.*, 1989, 1990).

Determination of the active site

Mutational analyses of TNF have been facilitated by the high TNF expression levels that could be reached in *Escherichia coli* cells. Furthermore, the fact that TNF does not form inclusion bodies but remains soluble in bacterial extracts, allows a fast and easy purification for wild type TNF as well as for many of the mutants. Lymphotoxin is much less soluble in *E.coli* lysates. However, efficient purification protocols (Schoenfeld *et al.*, 1991), together with optimalization of the ribosome binding site and adapted codons for *E.coli* throughout the gene (Seow *et al.*, 1989), have allowed lymphotoxin to be obtained in high quantities from bacteria.

The TNF and lymphotoxin mutants published so far are summarized in Table II. Many of the substituted residues are located in the interior of the monomeric and/or trimeric molecule. Not unexpectedly, any change in these usually highly conserved amino acids induces a structural distortion that indirectly causes inactivity. Drastic conformational disturbances can be detected by changes in solubility or immunoreactivity. Also, several techniques such as gel filtration, refolding behaviour, CD (circular dichroism), cross-linking, fluorescence and a combination of IR (infrared spectroscopy) with hydrogen exchange, have been used to evaluate the global conformation

Table I. Physicochemical characteristics of TNF and lymphotoxin

	TNF		LYMPHOTOXIN	
number of amino acids	human : 157 mouse : 156		human : 171 mouse : 169	
cysteines cystine-bridge	human : Cys69, Cys101 mouse : Cys69, Cys101 C69-C101	 Davis et al., 1987 Hsu et al., 1986 Wingfield et al., 1987	human : - mouse : Cys84	
N-glycosylation O-glycosylation	human : - mouse : present(Asn7)	Green et al., 1976 Haranaka et al., 1986 Jue et al., 1990	human: present(Asn62) mouse: present(Asn60) human: present(Thr7)	Aggarwal et al., 1985b Porter et al., 1990 Voigt et al., 1993
molecular weight (Da) : *theoretical* *SDS-PAGE* *cross-linking* *2D-ODD-IEF*[a] *sedimentation and ultracentrifugation* *small angle scattering* mol. weight 	17356 (monomer) 52068 (trimer) human : 17000 mouse : 15-18000 ±43000 55000 trimer composed of three slightly different subunits 49000 50000 51000 52000 54000 48000 50-53000	 Pennica et al., 1984 Kull and Cuatrecasas, 1984 Pennica et al., 1985 Haranaka et al., 1986 Browning and Ribolini, 1989 Lam et al., 1988 Smith and Baglioni, 1987 Eck et al., 1988 Schoenfeld et al., 1991 Wingfield et al., 1987 Sreekrishna et al., 1989 Smith and Baglioni, 1987 Arakawa and Yphantis, 1987 Schoenfeld et al., 1991 Yoshimura et al., 1988 Lewit-Bentley et al., 1988	18664 (monomer) 55992 (trimer) human : 18800-25000 ±50000 56000 46000 	 Aggarwal et al., 1984 Gray et al., 1984 Haas et al., 1989 Seow et al., 1989 Porter et al., 1990 Wakabayashi et al., 1990 Browning and Ribolini, 1989 Schoenfeld et al., 1991 Schoenfeld et al., 1991
crystallization *wild-type* *mutant A84V* *C69S*	trigonal (3.5 Å) rhombohedral (1.85 Å; twinned) tetragonal (<3 Å) trigonal (3 Å) cubic (3 Å) hexagonal (unstable) trigonal (2.9 Å) trigonal (2.2 Å) rhombohedral (1.8 Å; twinned)	Lewit-Bentley et al., 1988 Fiers et al., 1986 Eck et al., 1988 " Hakoshima and Tomita, 1988 " Jones et al., 1989 Saludjian et al., 1993 Van Ostade, unpubl. res.	hexagonal (1.9 Å)	Eck et al., 1992
Spectra *CD* *(circular dichroism)* *fluorescence (280 nm)* *IR-spectroscopy in combination with H-exchange*	near UV : max. : 280.5 nm : 285.5 nm : 291-292.5 nm far UV : min. : 219-221 nm max : 319 to 331 nm quenching with KI, AA[b] β-structure 35% α-helix 9% β-turns : 22% loops : 10%	Davis et al. 1987 Hsu et al., 1986) Narachi et al., 1987 Wingfield et al., 1987 Van Ostade et al., 1991 Srecktishna et al., 1989 Nishikawa et al., 1990 Narachi et al., 1987 Arakawa et al., 1990 Prestrelski and Arakawa, 1991	max. : 264 nm : 269 nm : 281 nm : 289 nm : 296 nm min. : 220 nm	Goh et al., 1992 Nishikawa et al., 1990 Wakabayashi et al., 1990 Nishikawa et al., 1990

isoelectric point	human : 5.2-5.9 : 5.8-6.8 mouse : 3.9 : 4.6-4.8	Aggarwal et al., 1985b Yamada et al., 1985 Geigert et al., 1987 Porter et al., 1990 Eck et al., 1988 Sreekrishna et al., 1989 Haranaka et al., 1986 Aggarwal et al., 1991a, 1991 Kull and Cuatrecasas ,1981	human : 5.8 : 6.9 : 8.3	Aggarwal et al., 1984 Porter et al., 1990 Hains and Aggarwal, 1989 Schoenfeld et al., 1991
reaction with : *diethylpyrocarbonate (DEP)* *N-Hydroxysuccinimidine ester*	concomitant activity reduction concomitant activity reduction	Yamamoto et al., 1987; 1989 Utsumi et al.,1991;1992 Klostergaard et al., 1992		
stability *within pH region*	5.5-10 4.8-10.5 and denaturation at 4.3	Haranaka et al., 1986 Wingfield et al., 1987	unstable in acidic conditions	Porter et al., 1990
detergents	relatively stable dissociation at 1% NP-40 stable at 2% NP-40	Porter et al., 1990 Browning and Ribolini, 1989 Lam et al., 1988	unstable stable at 1% NP-40	Porter et al., 1990 Browning and Ribolini, 1989
urea	denaturation at 3.8 M	Wingfield et al., 1987		
guanidinium-HCl	denaturation at 1.14 M	Wingfield et al., 1987		
protease sensitivity - trypsin - elastase - α-chymotrypsin - pepsine - cathepsin G - papain - *S. aureus* V8 - macrophage-bound proteases : N-aminopeptidase Ser-endopeptidase	+ - - - + + - - + - +	Aggarwal et al., 1985c Narhi et al., 1989 Haranaka et al, 1986 Scuderi et al., 1991 Bauvois et al., 1992 Haranaka et al., 1986 Aggarwal et al., 1985c Haranaka et al., 1986 " Scuderi et al., 1991 Bauvois et al., 1992 Haranaka et al., 1986 Aggarwal et al., 1985c Bauvois et al., 1992 "	±	Aggarwal et al., 1985b Scuderi et al., 1991 Aggarwal et al., 1985b Scuderi et al., 1991 Aggarwal et al., 1991
temperature 37°C-40 hr. 56°C-6 hr. 70°C-1hr. 100°C-2 min. 25°C-1 month 25°C-4 months 37°C-1 month	stable reduced activity no activity no activity reduced activity degraded degraded	Wingfield et al.,1987 " Green et al., 1976 Wingfield et al., 1987 Geigert et al. 1987 " "		

Except when indicated, data were derived from experiments with human TNF.
[a]2D-ODD-IEF: two dimensional, one dimension denatured isoelectric focusing.
[b]AA: acrylamide.

of TNF analogues. In addition, crystallization and X-ray diffraction of the inactive TNF mutant A84V (see below) is under way (Saludjian *et al.*, 1992). In the cases where loss of bioactivity was not accompanied by a gross structural alteration, the concerned residue was boxed and assumed to lie in or very near the active site (Figure 3a and b). Exceptions to this rule are residues S60 and H78 which are known to be positioned inside the molecule. Some mutations at these sites probably induce local conformational changes, leading to biological inactivity, but which are undetectable by physicochemical assays (Table II. Zhang *et al.*, 1992). The most relevant mutations, with respect to the TNF active site, will be discussed below.

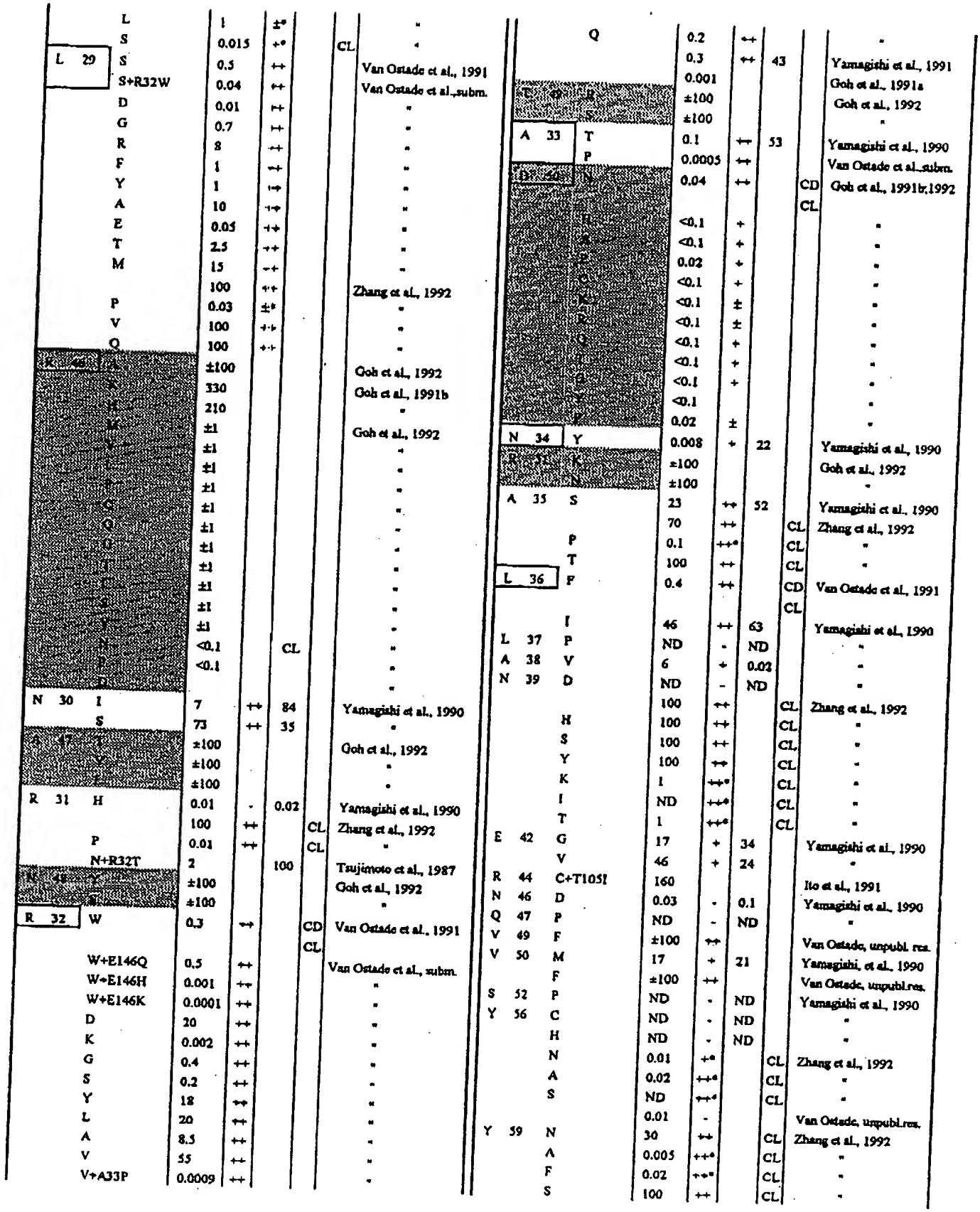
Importance of the N- and C-termini

Deletion at the N-terminus for eight amino acids increases the activity of TNF by a factor of 1.5–5, as measured in an L929

Table II. List of mutations in TNF and lymphotoxin

mutation	cytotox.	SDS GE	inter. react.	3D	ref.
wild-type	100	++	100		
26 kDa	< 0.1		+		Kriegler et al., 1988
					Perez et al., 1988
18.5 kDa	< 1.0	++			Cach and Beutler, 1989
wild-type+ R-I-R-M	> 100[a]				Soma et al., 1987
Δ1-7+P8M+S9H+ A10F+T26N	100		100		Kobayashi et al., 1986
Δ1-7+P8M+S9D+ A10P+T26N+Δ162-171	<10^{-4}				"
Δ1-2	150		+		Nakamura et al., 1990
	460				Kamijo et al., 1989
Δ1-2+L157F	10				"
Δ1-2+S3T+S4T+S5T	190				"
Δ1-4	150				Creasey et al., 1987
Δ1-5	150		±100		Tsujimoto et al., 1987
Δ1-6	±190				Nakamura et al., 1990
Δ1-25					Aggarwal et al., 1985b
Δ1-7	240				Creasey et al., 1987
	275		+		Nakamura et al., 1991b
	360		±100		Sidhu and Bollon, 1989
	350	++			Van Ostade, unpubl. res.
Δ1-7+P8R+S9K+D10R	600				Nakamura et al., 1991a
Δ1-7+R32E	0.03	+	+	GF	Nakamura et al., 1991b
Δ1-7+R32Y (mouse)	33	++			Van Ostade et al., subm.
Δ1-7+A35H	5				Masegi et al., 1989
Δ1-7+A84H	ND		+	GF	Nakamura et al., 1991b
Δ1-24	86 [b]			CD	Wakabayashi et al., 1990
				RF	
Δ1-24+Δ84-89	10^5 [b]			CD	"
				RF	
Δ1-8	170				Creasey et al., 1987
	400				Geigert et al., 1987
Δ1-25	±100	++			Nishikawa et al., 1990
Δ1-9	100	++			Mark et al., 1987
Δ1-26	100	++			Nishikawa et al., 1990
Δ1-10					Carlino et al., 1987
	10-35				Creasey et al., 1987
	33	++			Mark et al., 1987
	±60				Nakamura et al., 1991b
Δ1-27	±100	++		CD	Nishikawa et al., 1990
	110				Wakabayashi et al., 1990
Δ1-27+K86G+K89N+ Y95V	$4x10^4$				"
	4.5x10				"
Δ1-27+K89N+Y95N					
Δ1-11					
Δ1-28	ND	+			Nishikawa et al., 1990
Δ1-12	ND				Nakamura et al., 1991b
Δ1-17	ND				Nakamura et al., 1990
Δ1-17 + R-I-R-M	>wt				Soma et al., 1987
Δ1-20					
Δ1-37	ND				Nishikawa et al., 1990
Δ31-35+GSG	0.0005	++			Van Ostade et al., subm.
Δ137-157		-		PS	Gase et al., 1990; 1991
Δ137-138+Δ140-141		-		PS	"
Δ142-157					
Δ156-171	ND	-			Gray et al., 1984

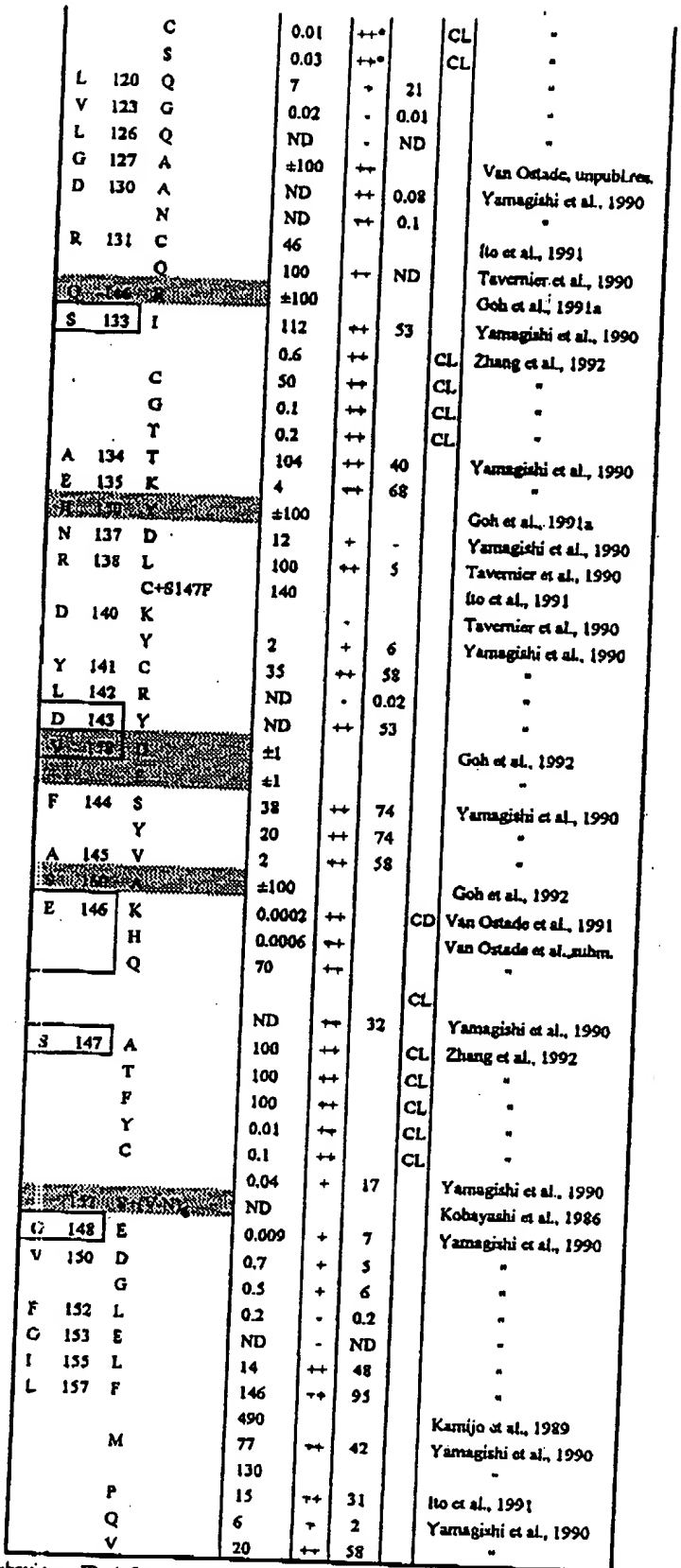
mutation	cytotox.	SDS GE	inter. react.	3D	ref.
Δ150-157	ND	-			Lin et al., 1992
Δ151-157	ND		ND	PS	Sidhu and Bollon, 1989
Δ155-156		-		PS	Gase et al., 1990; 1991
Δ155-157		-		PS	"
Δ156-157	ND		ND		Sidhu and Bollon, 1989
		-		PS	Gase et al., 1990; 1991
Δ157	ND	-			Lin et al., 1992
		-		PS	Gase et al., 1990; 1991
R 2 K+S4H+T7H	260				Kamijo et al., 1989
K+S4C+S5T +D10R	118	++			Soma et al., 1989
K+S4C+S5T +D10R+P12A	17	++			"
K+S5T+D10R	194	++			"
K+S5T+D10R +P12A	<17	++			"
S 4 C+S5T+D10R	165	++			"
C+S5T+D10R +P12A	<17	++			"
S 5 T+D10R	235	++			"
T+D10R+P12	<17	++			"
D 10 R	40				Ito et al., 1991
K 11 Q	±100	++			Zhang et al., 1992
M	±100	++			"
T	±100	++			"
N	±100	++			"
A 14 T	ND	-	0.01		Yamagishi et al., 1990
H 15 Y	0.004				Ito et al., 1991
	ND	-	0.01		Yamagishi et al., 1990
	0.005	++*		CL	Zhang et al., 1992
N	0.015	++*		CL	"
	0.0001				Yamamoto et al., 1987; 1989
Q	15				"
G	ND				"
K	ND				"
	ND				Van Ostade, unpubl. res.
D	0.01	++*			Zhang et al., 1992
P	0.001	++*			"
V 16 A	ND	-	0.01		Yamagishi et al., 1990
A 18 V	2	+	30		"
N 19 T	0.7	-	2		"
D 36 N	±100				Goh et al., 1992
H	±100				"
Y	50				Goh et al., 1991b
P 20					
F 37 T	±100				Goh et al., 1992
A	±100				"
S	±100				"
Q 21 L	46	++	68		Yamagishi et al., 1990
E 23 V	65	++	84		"
L 26 F	0.1	-	2		"
Q 27 P	±100	++			Zhang et al., 1992
L	±100	++			"
H	±100	++			"
K	±100	++			"
W 28 F	45	+		GF	Van Ostade et al., 1991
F+W114F	ND	-			Van Ostade, unpubl. res.
R	ND	-	ND		Yamagishi et al., 1990
C	100	++			Zhang et al., 1992



S	60	P	ND	-	ND		Yamagishi et al., 1990
		T	0.03	++		CL	Zhang et al., 1992
		A	0.07	++		CL	"
		F	0.016	+		CL	"
		Y	ND	++		CL	"
Q	61	L	ND	-	0.09		Yamagishi et al., 1990
V	62	D	ND	-	ND		"
		G	0.02	-	0.02		
F	64	S	0.01	-	0.03		"
C	69	S	62		±100		Tsujimoto et al., 1987
			20	++			Van Ostade, unpubl.res.
		S+C101S	40	++			Mark et al., 1987
		D+C101R	10			CD	Yamamoto et al., 1990
						FL	Prestrelski and Arakawa,
						DS	1991
						PS	
						RF	
						IR	
		A	±20				Goh et al., 1991a
		A+C101A	20		++	CD	Hsu et al., 1986
						FL	Narachi et al., 1987
						Q	
						GF	
		L+C101L	7		++	CD	Hsu et al., 1986
						FL	Narachi et al., 1987
						Q	
						GF	
P	70	L	77				Ito et al., 1991
T	72	Y	±20				Goh et al., 1991a
H	73	Q	33				Yamamoto et al., 1987
		Q+H78Q	33				Yamamoto et al., 1987, 1989
		Δ	75				Ito et al., 1991
L	75	P	1	+	22		Yamagishi et al., 1990
T	77	A	8	++	68		"
H	78	R	ND	-	0.002		"
		P	ND	++			Zhang et al., 1992
		Q	0.06	++			"
		L	100	++			"
T	79	A	112	++	63		Yamagishi et al., 1990
I	80	V	108	++	58		"
S	81	C	54	++	63		"
		R	5	++	63		"
I	83	F	23	++	54		"
A	84	V	ND	++		CD	Van Ostade et al., 1991
						CL	
						XR	Saludjian et al., 1992
		L	0.0006	++			Van Ostade et al., 1991
		I	0.002	++			"
		D	0.002	++			"
			ND	++	45		Yamagishi et al., 1990
S	105	A	±100				Goh et al., 1992
		T	±100				"
V	85	A	26.5	++	41		Yamagishi et al., 1990
		D	69.2	++	79		"
S	106	F	±100				Goh et al., 1992
		Y	±100				"
		D	±100				"
		A	±100				"
		L	±100				"

		N	±100				"
S	86	F	0.002	++		CD	Van Ostade et al., 1991
						CL	
		L	0.02	++			"
		V	0.4	++			"
		I	0.08	++			"
		A	26	++	69		"
Q	107	H	±1				Goh et al., 1992
		N	±100				"
Y	87	H	0.01	+	25		Yamagishi et al., 1990
			ND	++		CL	Zhang et al., 1992
		N	ND	++		CL	"
		S	ND	++		CL	"
Y	108	F	0.09			CD	Goh et al., 1992
						CL	
		C	<0.1				"
Q	88	L	15	++	46		Yamagishi et al., 1990
		R	14	++	43		"
P	109	A	±100				Goh et al., 1992
T	89	A	46	++	74		Yamagishi et al., 1990
K	90	R	54	++	84		"
V	91	D	0.0002	++		CD	Van Ostade et al., 1991
						CL	
		A	0.2	++			"
		I	2	++	37		Yamagishi et al., 1990
N	92	S	36	++	58		"
S	95	P	ND	-	0.01		"
		Y	0.5	++		CL	Zhang et al., 1992
		F	0.01	++		CL	"
		F+Q153V	52				Ito et al., 1991
S	110	T	±100				Goh et al., 1992
A	96	T	0.5	+	8		Yamagishi et al., 1990
S	99	N	5.0	+	27		"
P	100	H	46	++	74		"
		L	65	++	68		"
C	101	S	5	++	32		"
		A	±20				Goh et al., 1991a
		L	±20				"
Q	102	R	89	++	58		Yamagishi et al., 1990
		Δ	85				Ito et al., 1991
R	103	W	43	++	79		Yamagishi et al., 1990
T	105	P	17	++	34		"
P	106	S	160				Ito et al., 1991
		S+R131C	42				"
E	110	K	100	++	-		Yamagishi et al., 1990
K	112	M	92	++	90		Yamagishi et al., 1990
W	114	F	30	++		GF	Van Ostade et al., 1988
Y	115	C	1	++	40		Yamagishi et al., 1990
			0.004	++*		CL	Zhang et al., 1992
		F	0.03	++		CL	"
E	116	H	85				Ito et al., 1991
		V	6	++	74		Yamagishi et al., 1990
P	117	S	33				Ito et al., 1991
		L	0.07	-	10.0		Yamagishi et al., 1990
I	118	L	50	++	40		"
		V	22	++	53		"
Y	119	H	0.2	-	21		"
			0.1	++		CL	Zhang et al., 1992
		N	0.1	++		CL	"

Shaded mutations were carried out on the corresponding positions in lymphotoxin and the resulting activities were compared with wild type lymphotoxin. SDS−GE, control of the solubility and expression behaviour of the mutants by SDS gel electrophoresis; imm. react., control of the immunoreactivity of the mutants by reaction with a monoclonal anti-TNF antibody; 3-D, control of the global 3-D structure of the mutants by various physicochemical techniques; ND, not detectable; CD, circular dichroism; RF, refolding behaviour; GF, gel filtration; PS, protease sensitivity; CL, cross-linking, FL, fluorescence; Q,



		C	0.01	++*		CL	"
		S	0.03	++*		CL	"
L	120	Q	7	+	21		"
V	123	G	0.02	-	0.01		"
L	126	Q	ND	-	ND		"
G	127	A	±100	+-			Van Ostade, unpubl.res.
D	130	A	ND	++	0.08		Yamagishi et al., 1990
		N	ND	+-	0.1		"
R	131	C	46				Ito et al., 1991
		Q	100	+-	ND		Tavernier et al., 1990
G	132	[illegible]	±100				Goh et al., 1991a
S	133	I	112	++	53		Yamagishi et al., 1990
			0.6	++		CL	Zhang et al., 1992
		C	50	++		CL	"
		G	0.1	++		CL	"
		T	0.2	++		CL	"
A	134	T	104	++	40		Yamagishi et al., 1990
E	135	K	4	+-	68		"
P	136	[illegible]	±100				Goh et al., 1991a
N	137	D	12	+	-		Yamagishi et al., 1990
R	138	L	100	++	5		Tavernier et al., 1990
		C+S147F	140				Ito et al., 1991
D	140	K			-		Tavernier et al., 1990
		Y	2	+	6		Yamagishi et al., 1990
Y	141	C	35	++	58		"
L	142	R	ND	-	0.02		"
D	143	Y	ND	++	53		"
S	144	[illegible]	±1				Goh et al., 1992
		[illegible]	±1				"
F	144	S	38	++	74		Yamagishi et al., 1990
		Y	20	++	74		"
A	145	V	2	+-	58		"
S	145	A	±100				Goh et al., 1992
E	146	K	0.0002	+-		CD	Van Ostade et al., 1991
		H	0.0006	+-			Van Ostade et al., subm.
		Q	70	+-			"
						CL	
			ND	+-	32		Yamagishi et al., 1990
S	147	A	100	++		CL	Zhang et al., 1992
		T	100	++		CL	"
		F	100	++		CL	"
		Y	0.01	+-		CL	"
		C	0.1	++		CL	"
			0.04	+	17		Yamagishi et al., 1990
[illegible]		[illegible]	ND				Kobayashi et al., 1986
G	148	E	0.009	+	7		Yamagishi et al., 1990
V	150	D	0.7	+	5		"
		G	0.5	+	6		"
F	152	L	0.2	-	0.2		"
G	153	E	ND	-	ND		"
I	155	L	14	++	48		"
L	157	F	146	+-	95		"
			490				Kamijo et al., 1989
		M	77	+-	42		Yamagishi et al., 1990
			130				"
		P	15	+-	31		Ito et al., 1991
		Q	6	+	2		Yamagishi et al., 1990
		V	20	+-	58		"

fluorescence quenching; DS, dissociation behaviour; IR, infrared spectroscopy combined with H-exchange; XR, X-ray diffraction analysis. A deletion is represented by a Δ symbol and Δ1–7 indicates that the first seven residues were removed. An asterisk points to the presence of aggregated forms, seen on SDS gels. Positions at which mutants were clearly reduced in cytotoxic activity and maintained their immunoreactivity and/or physico- or biochemical characteristics are boxed. These residues are indicated on the 3-D structure in Figure 3a and b. Relative cytotoxic activity (wild type = 100%) was determined using the standard mouse L929 or WEHI assay (Ruff and Gifford, 1981; Espevik and Nissen-Meyer, 1986 respectively), except where indicated otherwise: (a) measured on the TNF-resistant human T24 cell line, (b) measured on the human WiDr cell line. However, no control experiments with TNF were performed on the latter cells so that the relatively high activity of the lymphotoxin mutants on WiDr cells can only be compared with wild type lymphotoxin and not with wild type TNF which tended to score higher in other systems in the same study (Wakabayashi *et al.*, 1990).

Fig. 2. Ribbon tracing of (a) the human TNF and (b) lymphotoxin trimers: Subunits are coloured red, yellow and blue. The disulphide bonds and cysteine residues in each monomer are visualized.

cytotoxicity assay (Creasey *et al.*, 1987; Geigert *et al.*, 1987; Tsujimoto *et al.*, 1987; Kamijo *et al.*, 1989; Sidhu and Bollon, 1989; Nakamura *et al.*, 1990; our unpublished results). After removal of nine residues, activity drops back to wild type levels (Mark *et al.*, 1987; Yamagishi *et al.*, 1990) and only after deletion of more than ten amino acids, is a decrease in activity observed (Carlino *et al.*, 1987; Creasy *et al.*, 1987; Mark *et al.*, 1987; Nakamura *et al.*, 1991b). A similar feature holds for lymphotoxin where up to 27 N-terminal amino acids could be removed without loss of biological activity (Aggarwal *et al.*, 1985b; Kobayashi *et al.*, 1986; Nishikawa *et al.*, 1990; Wakabayashi *et al.*, 1990). Note that Pro18 of lymphotoxin corresponds structurally to Val1 of TNF (Figure 1).

On the other hand, the C-terminus of TNF seems to be very sensitive, as deletion of eight, three, two and even one amino acids at this end drastically decreases solubility and bioactivity and augments the sensitivity to protease degradation (Sidhu and Bollon, 1989; Gase *et al.*, 1990, 1991). Also in lymphotoxin, the last 16 (Gray *et al.*, 1984) or ten residues (Kobayashi *et al.*, 1986) cannot be removed without drastically reducing the biological activity.

These results can be interpreted on the basis of the 3-D structure, as Lys11 in TNF (Lys28 in lymphotoxin) forms an ion pair with the C-terminal moiety of Leu157 (Leu71 in lymphotoxin) of the adjacent subunit (Sprang and Eck, 1990). Since the receptor binding site of TNF and lymphotoxin is localized at least in part in the lower half of the molecule (which is the broader half of the pyramidically shaped TNF trimer; see Figure 2a and b), removal of one of the terminal residues or deletion in their close proximity presumably leads to a disturbance of the local (and perhaps even more distal) conformation which could indirectly influence bioactivity. However, in contrast to this hypothesis is the observation of Zhang *et al.* (1992) who noticed that several substitutions at position 11 (K11Q, K11M, K11T, K11N) have no detectable effect on structure and biological activity. It is known that the N-terminus of both cytokines is very flexible and may even curl up and fold into the groove between the subunits (Sprang and Eck, 1992) where it can block the putative active site. Moreover, a murine TNF molecule with an additional ten amino acid stretch at the N-terminus, was devoid of cytotoxic activity (Cseh and Beutler, 1989). The requirement for rigidity of the active site environment is further supported by the observation that substitution of Leu157 by Phe, Met or Gln increases bioactivity (Kamijo *et al.*, 1989; Yamagishi *et al.*, 1990), probably by increasing tighter hydrophobic intersubunit interactions.

A TNF mutant shortened by 17 N-terminal amino acids has proven to be inactive (Nakamura *et al.*, 1990). It is therefore a remarkable claim that addition of the basic amino acid stretch Arg−Ile−Arg−Met at this Δ17 mutant would confer on the molecule an even broader cytotoxicity *in vitro* as well as *in vivo* (Soma *et al.*, 1987). Furthermore, the replacement of Asp10 by

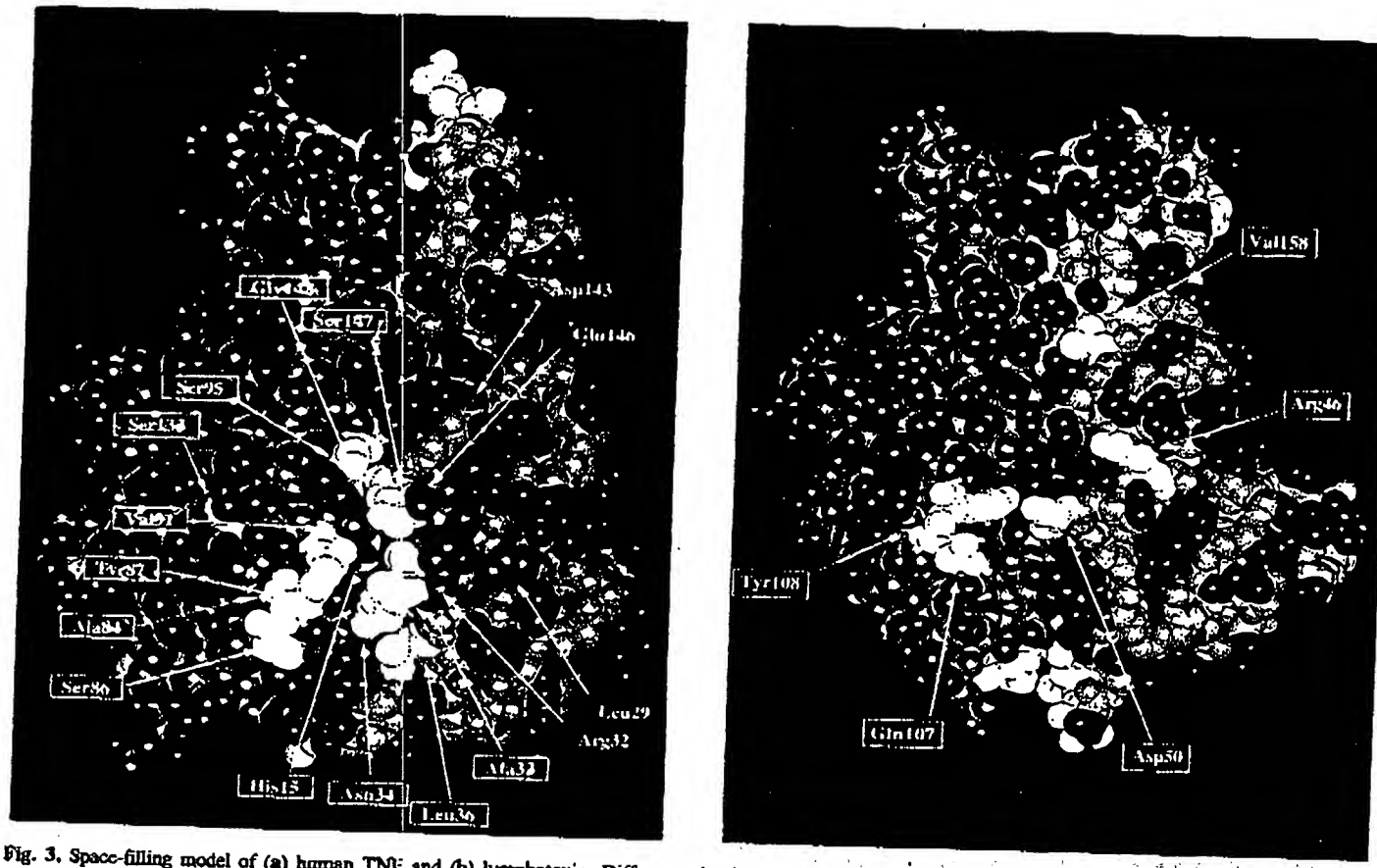


Fig. 3. Space-filling model of (a) human TNF and (b) lymphotoxin. Different subunits are represented in red, yellow and blue. Side chain atoms of the two subunits in front are of darker colour. Relevant amino acids (Cα atoms and side chains) which are presumed to be involved in receptor interaction (see Table II) are labelled and marked with white. Side chains that preferentially interact with the TNF-R75 or TNF-R55 are coloured green and magenta respectively.

arginine (=D10R mutation) also caused a broader cytotoxic character, together with the maintenance of solubility and a slightly increased cytotoxicity towards L929 cells (Soma *et al.*, 1988). However, since the sp. act. of the control wild type TNF on these cells was approximately ten times lower than generally accepted (1.7×10^6 versus $1-5 \times 10^7$ U/mg), the extent of the increase in activity of the mutants towards sensitive and more resistant cells should be re-evaluated and compared to international standardized wild type TNF. Another group reported the inability of the D10R mutant to kill wild type TNF-resistant cell lines, one of which (the bladder carcinoma T24) was the same as in the previous study (Ito *et al.*, 1991) and similar site-specific mutations in the N-terminal region (R2K-S4H-T7H, Kamijo *et al.*, 1989; P8R-S9K-D10R, Nakamura *et al.*, 1991a) only caused a very small increase in L929 cytotoxicity. It may be noted that cell lines used in different laboratories under the same name, may vary considerably in sensitivity and the use of appropriate standard TNF preparations is highly recommended.

Mutagenic analysis

Because originally very few indications were available concerning the location of the active site on the TNF molecule, random mutagenesis on the gene was performed and inactive molecules were selected on the basis of their cytotoxic activity against mouse L-M cells (Yamaghishi *et al.*, 1990) or mouse L929 cells (Van Ostade *et al.*, 1991). Starting from these results, additional site-specific mutations were then introduced. The conformation of the resulting TNF analogues was investigated by analysis of solubility and immunoreactivity (Yamagishi *et al.*, 1990) or their solubility, circular dichroism spectra and trimerization properties

Fig. 4. Ribbon tracing of a bottom–top view of the TNF structure. Different subunits are coloured red, yellow and blue and amino acids, putatively involved in receptor interaction are marked white. To make the triplicate active site clearly visible, part of the bottom region was cut off.

(Van Ostade *et al.*, 1991). Results from these two independent studies were quite similar: TNF activity dropped considerably when alterations were introduced in regions 29–36, 84–91, 117–119 and 143–148, without marked change in immunoreactivity or physicochemical characteristics. More specifically, substitutions on the following positions reduced TNF cytotoxicity by a factor of > 100: 29, 32, 33, 34, 36, 84, 86, 87, 91, 117, 119, 143, 146, 147 and 148 (Table II). Except for region 117–119, which is involved in the formation of an inner β-sheet, the other three stretches are all located in loop structures which cluster in the 3-D model of TNF around the cleft, located each time between two subunits of the trimer (Figure 3a and b). The majority of the aforementioned residues are positioned in the lower half of the molecule, near the base of the pyramidal structure, at the outer surface. By convention, the three-sided pyramid is represented with its tip upwards, but when bound to its receptor, the tip is oriented towards the cell surface (Banner *et al.*, 1992).

It is not easy to evaluate to what extent an amino acid substitution in these regions alters the biological activity in either a direct or an indirect manner. Indirect inactivation by destabilizing subunit contacts and, thus, by altering the conformation of the active site cleft, is quite conceivable (Sprang and Eck, 1992). However, Tyr87, for example, is a protruding residue, not involved in intersubunit contacts (Figure 4) and substitution by histidine, asparagine or serine reduces the cytotoxicity drastically (Yamagishi *et al.*, 1990; Zhang *et al.*, 1992). Moreover, the same residue is also involved in the active centre of lymphotoxin where even a conservative change to phenylalanine results in a more than 1000-fold drop in activity (Goh *et al.*, 1992). Hence, Tyr87 could form a direct contact with the TNF receptor, although it has been reported that iodination of TNF and lymphotoxin on this Tyr residue did not reduce the activity (Aggarwal *et al.*, 1985a). Another potential candidate for direct interaction with the receptor is Asp50 in lymphotoxin (position 33 in TNF) because of the large decrease in bioactivity after substitution by conservative amino acids such as asparagine or glutamic acid (Goh *et al.*, 1992). The side chain of this residue clearly points to the exterior (Figure 3b) and, hence, is able to form a direct contact with the receptor. However, the direct involvement in the active centre of TNF of the loop containing Asp50 is still unresolved. Zhang *et al.* (1992) constructed several mutations in this region (L29M, L29V, L29Q, L29P, R31P, R31H, A35P and A35T). They concluded that only the rotation-restricted proline substitutions were able to reduce considerably receptor binding and cytotoxic activity, probably as a result of a local modified conformation of the polypeptide backbone. These results suggest that direct receptor binding sites are more distantly located from the loop connecting β-strands a and a'.

Another random mutagenesis study revealed two potentially relevant TNF muteins: H15Y and P117S (Ito *et al.*, 1991). Although no analysis was made regarding the possible change in conformation of these mutants, the two positions agree well with other reports (position 15, Yamamoto *et al.*, 1987, 1989; position 117, Yamaghishi *et al.*, 1990).

It may be noted that although the concentration of the inactivating mutations in a single area constitutes an intrinsic control for the random experiments, the possibility is not excluded that other domains of the molecule are also involved in receptor contact but were not revealed due to conformational disturbances which some substitutions might cause. Thus, while the lower half of the TNF molecule is certainly involved in receptor interaction, these data do not exclude the possibility that additional sites also play a role. Furthermore, caution must be taken since activity determinations were performed on mouse cell lines (L929 and WEHI) while a species-specific effect can clearly be observed with the majority of the muteins when tested on human cell lines (e.g. Hep-2 and KYM) (Van Ostade *et al.*, submitted). This indicates that the heterologous ligand–receptor interaction is more restricted and, hence, more sensitive to mutagenesis, as compared to the homologous interaction.

Consistent with the aforementioned results are the data of Masegi *et al.* (1990) who showed that mutagenesis in two of the previously mentioned loops (R32E and A84H) could reduce the cytotoxic activity by a factor of 10^4. Zhang *et al.* (1992) also obtained evidence for the possible involvement of Ser95, Ser133 and Ser147, the first of which also lines the intersubunit cleft. Ser133 is located outside the groove and this may suggest an extended receptor binding surface, at least in the lower half of the molecule. However, the activity data of Zhang *et al.* (1992) are in contrast to those reported by Ito *et al.* (1991) and Yamagishi *et al.* (1990) on S95F+G153V and S133I mutants respectively (Table II). Also conflicting results concerning the involvement of the R31H and N39D mutants exist in the reports of Zhang *et al.* (1992) and Yamagishi *et al.* (1990) (Table II). The explanation for these contradictions is still unclear but measurement based on purified proteins versus bacterial lysates could be a possible reason.

Other studies by site-specific mutagenesis were directed at some conserved residues in the primary structures of the two cytokines. For instance, the three histidine residues (positions 15, 73 and 78) of which the first and the last are conserved in TNF and lymphotoxin, were changed to various other amino acids (Table II). Only mutagenesis of His15 caused a clear decrease in bioactivity, but all the mutants (H15N, H15Q, H15K, H15G) accumulated in bacterial inclusion bodies and had to be solubilized by a denaturation–renaturation step (Yamamoto, 1987, 1989). As a consequence, the possibility that inactivity is a result of incorrect refolding of the His15 mutants cannot be excluded. Moreover, Zhang *et al.* (1992) suggested that the putative hydrogen bond between His15 and Tyr59 or the local polar environment in this area is necessary for a correct folding of the monomer. The two tryptophan residues (positions 28 and 114) are not only conserved in all TNF species but also in lymphotoxin (Figure 1). It turned out, however, that replacement by phenylalanine had no pronounced effect on cytotoxoic activity (Van Ostade *et al.*, 1988). Also substitution of Trp28 by cysteine did not alter bioactivity, although the mutants W28R, W28L and W28S resulted in abnormal aggregated forms, suggesting a structural role for Trp28 (Zhang *et al.*, 1992). The two cysteines (69 and 101) are conserved in all TNF species and even in bovine and rabbit lymphotoxin but not in human lymphotoxin (Figure 1). As mentioned before, these residues form an intramolecular disulphide bridge, thereby connecting two loops on top of each subunit. This bond was broken by replacing one (Tsujimoto *et al.*, 1987; Yamagishi *et al.*, 1990; Lin, 1992; unpublished results) or the two Cys residues (Mark *et al.*, 1987; Narachi *et al.*, 1987; Arakawa *et al.*, 1990; Prestrelski and Arakawa, 1991; Lin, 1992). Only a slight reduction in activity was observed in each case and it was reported that the mutant adapted a looser, more flexible structure in solution, as compared to native TNF (Arakawa *et al.*, 1990; Prestrelski and Arakawa, 1991). Moreover, it was observed that crystals, grown either from reduced TNF (Eck *et al.*, 1988) or from the disulphide bridge-deficient mutant C69S (unpublished results) diffract to a high

resolution, but unfortunately were twinned and, thus, unsuitable for X-ray diffraction analysis. Also deletion of a neighbouring amino acid (Gln 102) had no influence on cytotoxic activity (Ito *et al.*, 1991).

Other evidence regarding the structure–function relationship

Some theoretical considerations were suggestive for an active centre in the lower half of the molecule. Jones *et al.* (1990b) were able to project onto the TNF structure receptor-interacting residues of viral coat proteins, having a structural similarity to TNF (e.g. picornavirus HRV14 and the foot-and-mouth disease virus FMDV). In the case of Ser133 and the tripeptide Lys128–Gly129–Asp130, even some sequence homology with the corresponding viral residues was noted and, in addition, Ile83 was also a serious candidate. Mutations in the much more related CD40 ligand (see above) that cause non-functional molecules, leading to the disorder of X-linked immunodeficiency with hyper IgM, map onto positions 13 (DiSanto *et al.*, 1993) 18, 19, 130 (Aruffo *et al.*, 1993), 28 (Korthäuer *et al.*, 1993) 43 and 122 (Allen *et al.*, 1993) when projected onto the TNF structure. All these mutations are drastic, non-conservative replacements that are located in the lower half of the molecule and probably disrupt the conformation of the CD40 binding site.

Another approximate localization of the active site came from the determination of the epitopes of neutralizing antibodies. Antibodies were raised against several synthetic TNF fragments and were able to react with the native molecule. However, only antibodies against the N-terminal sequences 1–15 and 1–31 had neutralizing potency while antibodies to epitopes in the middle/top region did not (Socher *et al.*, 1987; Corti *et al.*, 1992b). In a similar study, the last 31 residues of human TNF were replaced by their corresponding mouse amino acids. Such a TNF chimera completely lost a binding site for a neutralizing antihuman monoclonal antibody. Substitution of the only three conserved residues in this stretch, pointed to Arg131 as being involved in the epitope but not in the active centre. As the antibody was neutralizing, residues essential for bioactivity must be located in close proximity to this Arg131 (Tavernier *et al.*, 1990).

Finally, another strategy for active site determination involved the introduction of consensus recognition sequences for N-linked glycosylation in the loop regions of the protein. Expression in Chinese hamster ovary cells (CHO cells) resulted in rationally designed, glycosylated human TNF molecules. These studies learned that introduction of carbohydrate groups on loop regions at the bottom (loops 6–11, 30–34, 38–42, 52–57, 85–89, 125–129 and 155–158), blocked the active centre of TNF. Linkage of glycosyl groups to loops, positioned higher in the molecule had no effect (Leung and Leung, 1992).

Mutant TNF molecules that differentially bind to the two TNF receptors

Two TNF receptors (TNF-R55 and TNF-R75) from human (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990) and mouse (Barrett *et al.*, 1991; Goodwin *et al.*, 1991; Lewis *et al.*, 1991) have now been cloned. The mouse TNF-R75 does not bind human TNF (Lewis *et al.*, 1991; Tartaglia *et al.*, 1991) and this explains the decreased activity of human TNF towards murine thymocytes or T-cell lines (Plaetinck *et al.*, 1987; Ranges *et al.*, 1988, 1989) which express this receptor type as the major form.

Early mutagenesis studies already suggested a differential activity of some TNF muteins, maintaining the wild type activity in one assay system but showing a reduced activity in another. The double cysteine mutant, C69A–C101A, had an antiviral activity on HeLa cells comparable to the native protein, but cytolytic activity and inhibition of intracellular lipid production was reduced by a factor of 5–7 (Narachi *et al.*, 1987). Also another double mutant, R31N–R32T, exhibited wild type growth enhancing activity on diploid fibroblast cells, whereas cytotoxic activity towards a myorhabdosarcoma cell line was reduced ±60-fold (Tsujimoto *et al.*, 1987). On the other hand, changing Leu157 (the last residue in TNF and lymphotoxin) to phenylalanine, resulted in a mutant that was five and 20 times more potent in the induction of macrophage differentiation and cytotoxicity respectively, compared to wild type TNF (Kamijo *et al.*, 1989). Although such results have to be evaluated with caution considering the inaccuracy of many cell biological assays, they can be interpreted as cell-specific responses to a signal mediated by one TNF receptor or as differential binding of TNF towards the two TNF receptors TNF-R55 and TNF-R75. The latter possibility was strengthened by the observation that a neutralizing anti-TNF antibody inhibited cytotoxicity and receptor interaction in one tumour model but not in another (Rathjen *et al.*, 1991). We have characterized in detail two TNF mutants, L29S and R32W, which have maintained almost completely the ability to bind to TNF-R55, while the TNF-R75 affinity was decreased drastically (Van Ostade *et al.*, 1993). In addition, this difference in physical binding behaviour towards the two TNF receptors was supported by the biological activities they mediated. In a subsequent study, two mutations at position 29 (L29G and L29Y) were shown to be even more discriminative, as compared to L29S. Also charge reversal at position 146 (E146K) results in a marked differential binding and activity. The results obtained thus far indicate that the TNF-R75-specific region is located in a cluster of three residues at the right side of the groove (Figure 3; Van Ostade *et al.*, submitted). Such an uncoupling of TNF-R55- and TNF-R75-mediated activities could have an interesting potential regarding the clinical use of TNF as an anticancer agent. Indeed, *in vivo* toxicity of human TNF in normal mice is ~50 times lower, compared to mouse TNF, while selective tumour toxicity remained unaffected (Brouckaert *et al.*, 1992), suggesting a special role for TNF-R75 in TNF-induced systemic toxicity. Since in the human system the TNF-R55-specific mutants L29S, R32W and E146K could act as the equivalent of human TNF in mice, they offer the prospect of reduced general toxicity, when used in cancer patients. In addition, the R32W and E146K mutants show a decrease in activity towards neutrophils and endothelial cells (Barbara *et al.*, in preparation), two cell types which are believed to play an important part in TNF-induced systemic toxicity (Beutler and Cerami, 1988). Uncoupling of TNF-R55- and TNF-R75-mediated activities was also possible in the mouse system (mouse R32Y; Van Ostade *et al.*, submitted) and, therefore, offers the possibility of testing the efficacy of differentially binding muteins *in vivo* in mice (unlike human TNF, these mouse TNF mutants are expected to be almost non-immunogenic). Furthermore, a reversed differential binding (affinity for TNF-R75 less affected as compared to TNF-R55) and concomitant receptor-mediated activity was observed with several substitutions at position 143 in human TNF (Van Ostade *et al.*, submitted). It thus appears now that both TNF receptors can interact, in addition to a common region, with at least one specific area on the ligand (Figure 3).

Difference between TNF and lymphotoxin

Certain cell types respond in a different way to TNF and to lymphotoxin (Aggarwal, 1991b and references therein; Porter,

1990 and references therein; Wakabayashi *et al.*, 1990). Both cytokines bind to the purified receptors with similar affinities (Schoenfeld *et al.*, 1991) although on some cell lines a slightly higher affinity for TNF was observed (Aiyer and Aggarwal, 1990; Andrews *et al.*, 1990; Desch *et al.*, 1990; Espevik *et al.*, 1990; Wakabayashi *et al.*, 1990; Raitano *et al.*, 1991). Also remarkable is the weaker binding of lymphotoxin towards the soluble receptor forms, particularly towards TNF-R55 (Seckinger *et al.*, 1989; Engelmann *et al.*, 1990b; Garanaga *et al.*, 1990; Kohno *et al.*, 1990; Loetscher *et al.*, 1991; Pennica *et al.*, 1992). Obviously, it means that the interaction between the (soluble) receptor and the ligand cannot be identical in molecular detail for TNF and for lymphotoxin. In this respect, it is noteworthy that after alignment with TNF, a deletion and an insertion in lymphotoxin fall within two loops at the top of the trimer (Figure 1; Eck *et al.*, 1992). These loops give the lymphotoxin structure a more flattened (due to the absence of the 103–108 TNF stretch, which is the highest protruding loop in the TNF molecule) and a fan-shaped outlook (insertion of four amino acids between loop residues 70 and 71 in TNF) (Figure 2a and b). Shortening of the latter loop in lymphotoxin (deletion of residues 84–89, lymphotoxin numbering) and, thus, making the molecule more 'TNF-like', resulted in a mutant that apparently showed no drastic change in the other parts of the structure, as determined by circular dichroism spectra and refolding experiments (Wakabayashi *et al.*, 1990). However, this deletion mutant had a remarkably increased activity, corresponding to a higher receptor binding affinity and amino acid replacements in this region could only partially mimic the effect of the deletion. On the other hand, in those assays where TNF was used for comparison, none of the lymphotoxin mutants reached the TNF potency (Wakabayashi *et al.*, 1990). More activity and affinity studies on cells that respond differently to TNF and lymphotoxin need to be done before one can conclude that a deletion in this loop indeed makes lymphotoxin more 'TNF-like' not only in structure but also in activity. If confirmed, the receptor interaction site of both molecules may have to be extended upward.

Thus far, evidence for involvement in the active site of lymphotoxin only points towards residues 50 and 108 and, to a lesser extent, to positions 46, 107 and 158 (lymphotoxin numbering), since substitution at these sites caused a reduction in bioactivity, corresponding with decreased receptor binding (Goh and Porter, 1991, 1992). After superposition onto the TNF structure, these sites (29, 33, 86, 87 and 143 in TNF numbering) are indeed located in loop regions 29–36, 82–91 and 143–148, lining the cleft between the two subunits. In contrast with TNF, however, alterations in neighbouring residues (amino acids 47, 48, 49, 51, 105, 106, 109 and 160; lymphotoxin numbering) did not affect bioactivity. Possibly, the active site of lymphotoxin is not as extended as it is in TNF. Alternatively, the active site region in TNF could be more rigid, such that amino acid substitutions could more easily induce a conformational distortion, indirectly leading to loss of activity.

The ligand–receptor complex

Not much has been published thus far regarding the 3-D structure of the two TNF receptors. Amino acid sequence comparison allowed a relationship between the external parts of both molecules and those of the nerve growth factor receptor (NGF-R) to be established. Each contains three or four subdomains, characterized by a strongly conserved pattern of cysteine residues (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). The fourth domain (most distal from the cell membrane) of TNF-R55 seems to be important, although not essential for TNF and lymphotoxin binding, but the first domain is highly necessary (Marsters *et al.*, 1992). The fact that the overall charges of the ligand and of either receptor are opposite (negative and positive respectively) could be an indication of important electrostatic interactions. Since the active form of TNF and lymphotoxin is a trimer (Figure 4) (Arakawa and Yphantis, 1987; Smith and Baglioni, 1987; Wingfield *et al.*, 1987; Lewit–Bentley *et al.*, 1988; Schoenfeld *et al.*, 1991), it follows that binding results in receptor clustering (Loetscher *et al.*, 1991; Pennica *et al.*, 1992). For most if not all TNF activities, this process is sufficient for signal triggering, since monoclonal antibodies (especially IgM) against either of the two receptors were able to mimic TNF activities (Engelman *et al.*, 1990a; Espevik *et al.*, 1990; Shalaby *et al.*, 1990; Tartaglia *et al.*, 1991; Tartaglia and Goeddel, 1992; Vandenabeele *et al.*, 1992). The complex, formed by the attachment of three TNF-R55 receptors to one lymphotoxin trimer, has a rod-like structure (Loetscher *et al.*, 1991) and preliminary X-ray diffraction data indicate that the four receptor subdomains are aligned and interact with discrete regions on the ligand, which points with its top towards the cell membrane (Banner *et al.*, 1992).

Has TNF other active centres?

Interaction with membranes

Thus far, it has been accepted that the active site of TNF was identical to the receptor binding site. Some claims, however, suggesting an additional function of the cytokine have been made. Dependent on lipid composition, TNF concentration and pH, association of TNF with or integration in artificial membranes was demonstrated (Baldwin *et al.*, 1988; Debs *et al.*, 1989; Roozemond *et al.*, 1989). Moreover, high TNF concentrations at low pH values induce an increase in permeability of phospholipid vesicles (Oku *et al.*, 1987), a process that can occur synergistically with interferon-γ (Yoshimura and Sone, 1987) and could be the result of integration of TNF into the membrane. In addition, Kagan *et al.* (1992) showed that under approximately the same conditions, the conductance of planar phospholipid membranes increased, which was explained by assuming that Na^+ ions could pass the membrane by flowing through the central channel of inserted intact TNF trimer molecules. How this lipid penetration process takes place is still an open question, but the maintenance of bioactivity, together with decreasing immunoreactivity (Debs *et al.*, 1989), increasing hydrophobicity (possibly by the surface exposure of the two tryptophan residues) and increasing sensitivity to endopeptidase C (Kagan *et al.*, 1992) indicates that conformational rearrangements could occur. Lysosomal membranes are possible targets for this process since receptor-bound TNF becomes internalized and accumulates in the acidic environment of the lysosomes. It has been shown by Ohsawa and Natori (1988) that, after TNF treatment, only TNF-sensitive cells release a permeability-inducing activity for liposomes in the medium. The authors proposed that a specific, TNF-degrading protease was present in these cells and that a resulting degradation product was detected in the medium. Finally, the peripheral localization and partial periplasmatic accumulation of TNF, after its expression in *E.coli* cells, has also been suggested as an argument for an interaction of TNF with membranes (Gase *et al.*, 1991).

One must, however, keep in mind that all well-characterized TNF mutants with reduced biological activity (cytotoxic as well as mitogenic and gene induction activities) show also a drop in

receptor affinity. Furthermore, since thus far there is no report in which a TNF biological effect could not be mimicked by agonistic monoclonal antireceptor antibodies, it may be concluded that, for all the major functions of TNF, receptor clustering explains fully the mechanism of action. However, the possibility that TNF membrane insertion could mediate an as yet undiscovered, biological function is not excluded.

Lectin-like binding

In addition to the properties mentioned above, TNF under acidic conditions also shows an affinity for high mannose-substituted glycoproteins (Moonen *et al.*, 1988; Muchmore *et al.*, 1990). It is not known whether this binding is biologically relevant.

Activity of peptide fragments

In analogy with the immunostimulatory activity of synthetic peptides from interleukin-1 (Antoni *et al.*, 1986; Mosley *et al.*, 1987), a peptide stretch (positions 31–68) of TNF showed some weak chemotactic stimulatory activity towards fibroblast cells (Postlethwaite and Syer, 1990), but was 10^4-fold less potent than wild type TNF. Despite this very low activity, it remains intriguing that the first part of this peptide (amino acids 31–53) shows a clear monocyte-stimulating activity (Steimer *et al.*, 1992), while the second part (positions 54–68) has some neutrophil-activating capacity (Rathjen *et al.*, 1992). In addition, a cyclic hexapeptide, corresponding to amino acids 127–132 of murine TNF, was reported to cause a very weak cytotoxicity towards several tumour cell lines (Sheh *et al.*, 1990). Obviously, in these cases the signal could not be transmitted through receptor clustering. Whether or not these observations are relevant to an understanding of the true *in vivo* mechanism of action of TNF remains to be proven.

TNF hybrids

The genetic coupling of lymphotoxin (mutant Δ1-23) or TNF (mutant S5T + D10R) mutants to interferon-γ (Feng *et al.*, 1988) and thymosin-β4 (Tsuji *et al.*, 1989) respectively, resulted in chimerae with an increased or unchanged cytotoxic activity *in vitro* respectively. The augmentation of cytotoxicity of the former construct possibly resulted from the synergistic effect, well known for these cytokines (Sugarman *et al.*, 1985; Fransen *et al.*, 1986). The usefulness of the TNF/thymosin-β4 hybrid was further investigated in tumour-bearing mice (Noguchi *et al.*, 1991). Also a chimeric human/mouse antitransferrin receptor antibody-Fab fragment was linked to human TNF and showed cytotoxic activity as well as transferrin receptor binding characteristics on tumour cell lines (Hoogenboom *et al.*, 1991a, b). Although these constructs had apparently retained immunoreactivity with at least some anti-TNF monoclonal antibodies, the actual oligomeric state of the TNF part remains to be elucidated and caution must be used in the interpretation of the activity data. More particularly, one wonders what kind of structure results from coupling the TNF polypeptide, which normally trimerizes, to an interferon-γ or an immunoglobulin heavy chain polypeptide, which normally dimerizes. Also immunogenicity of such chimerae would be a cause of concern.

Conclusion

At least part of the receptor binding site of TNF is located in the lower half of the conically shaped trimeric structure. This can be concluded not only from mutagenesis analysis, but also from deletion studies at the N- and C-terminal ends, blocking experiments with antibodies or carbohydrate groups and from comparison with the receptor binding sites of related viral coat proteins. Random and site-specific mutagenesis showed that amino acids involved in receptor binding of TNF, are located at each interface between two subunits, particularly loops 29–36, 84–91 and 143–148. Consistent with these observations are the positions of the active site residues of lymphotoxin, since they correspond with the three loops in TNF after superposition of the two structures. The groove between the subunits is repeated three times in the TNF and lymphotoxin trimer. Hence, one TNF or lymphotoxin molecule has three receptor binding sites and acts by clustering three receptors, an event that is sufficient, at least for the large majority of TNF effects, to transmit the TNF signal. Also, TNF mutants that bind selectively to one of the two receptors have been developed. Considering that the intracellular domains of the two TNF receptors are totally unrelated and that the two receptors have a different cell distribution and regulation, these receptor-restricted TNF mutants offer the potential to uncouple several TNF-mediated effects. More specifically, based on *in vivo* results in the murine system, one may hope that TNF mutants which only react with TNF-R55 would have retained the beneficial antitumour effects, but be at least partially devoid of *in vivo* deleterious effects. Detailed information at the atomic level regarding the interaction of TNF with either of its two receptors has yet to come from crystal structures of ligand–receptor complexes. This, together with further protein engineering, should permit an understanding of the fine details of TNF and lymphotoxin and their interactions with their two receptors.

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Osteoprotegerin: A Novel Secreted Protein Involved in the Regulation of Bone Density

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Summary

A novel secreted glycoprotein that regulates bone resorption has been identified. The protein, termed Osteoprotegerin (OPG), is a novel member of the TNF receptor superfamily. In vivo, hepatic expression of OPG in transgenic mice results in a profound yet nonlethal osteopetrosis, coincident with a decrease in later stages of osteoclast differentiation. These same effects are observed upon administration of recombinant OPG into normal mice. In vitro, osteoclast differentiation from precursor cells is blocked in a dose-dependent manner by recombinant OPG. Furthermore, OPG blocks ovariectomy-associated bone loss in rats. These data show that OPG can act as a soluble factor in the regulation of bone mass and imply a utility for OPG in the treatment of osteoporosis associated with increased osteoclast activity.

Introduction

Bone is a complex tissue composed of cells, collagenous matrix, and inorganic elements. It provides many essential functions, including mechanical support, protection of vital organs, a microenvironment for hematopoiesis, and a depot for calcium and other minerals. The growth, development, and maintenance of bone is a highly regulated process (Nijweide et al., 1986). The level of bone mass reflects the balance of bone formation and resorption, which at the cellular level involves the coordinate regulation of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Osteoblasts arise from mesenchymal stem cells and produce bone matrix during development, after bone injury, and during normal bone remodeling that occurs throughout life. In contrast, osteoclasts differentiate from hematopoietic precursors of the monocyte–macrophage lineage and resorb bone matrix. Both these cell types are influenced by a wide variety of hormones, inflammatory mediators, and growth factors (Suda et al., 1992; Mundy, 1993a, 1993b). An imbalance of osteoblast and osteoclast functions can result in skeletal abnormalities characterized by increased (osteopetrosis) or decreased (osteoporosis) bone mass.

The study of osteopetrosis in mutant mice has led to significant advances in the understanding of the processes that regulate bone mass (Marks, 1989). From these studies we have learned the following: (i) genetic defects in osteoclast development, maturation, and/or activation lead to decreased bone resorption and uniformly result in severe osteopetrosis (Marks, 1989); (ii) the stromal microenvironment plays an essential role in osteoclast differentiation (Udagawa et al., 1989); (iii) signal transduction from the cell membrane through Src tyrosine kinase is necessary for osteoclast-mediated bone resorption (Soriano et al., 1991); and (iv) changes in gene expression patterns regulated by the nuclear factor Fos play an important role in osteoclast development (Wang et al., 1992; Grigoriades et al., 1994). Additionally, osteoclast maturation and activation are regulated by osteoblast-derived factors during remodeling (Rodan and Martin, 1981). The disregulation of osteoblast and osteoclast functions in these systems implicates genes that act as key determinants in the regulation of bone mass. However, relatively little is known about the soluble factors that act physiologically to regulate osteoclast development.

This report describes the isolation of a determinant in the regulation of bone mass, a novel secreted member of the tumor necrosis factor receptor (TNFR) superfamily. The TNFR superfamily consists mostly of transmembrane proteins that elicit signal transduction in a variety of cells. This novel member lacks any apparent cell-association signals, however, indicating it likely acts in the extracellular milieu. Transgenic mice expressing this secreted protein exhibit a generalized increase in bone density (osteopetrosis) associated with a decrease in osteoclasts. We named this protein Osteoprotegerin (OPG), i.e., to protect bone. Administration of recombinant OPG produces similar effects in normal mice and protects against ovariectomy-associated bone loss in rats. In vitro, OPG blocks osteoclastogenesis in a dose-dependent manner. OPG appears to block the differentiation of osteoclasts, the principal if not sole bone-resorbing cell type, suggesting that it can act as a humoral regulator of bone resorption.

Results

Identification and Isolation of a Novel Secreted TNFR-Related Protein

OPG was first identified by sequence homology as a possible novel member of the TNFR superfamily during a fetal rat intestine cDNA-sequencing project. A full-length version of this clone was isolated and sequenced

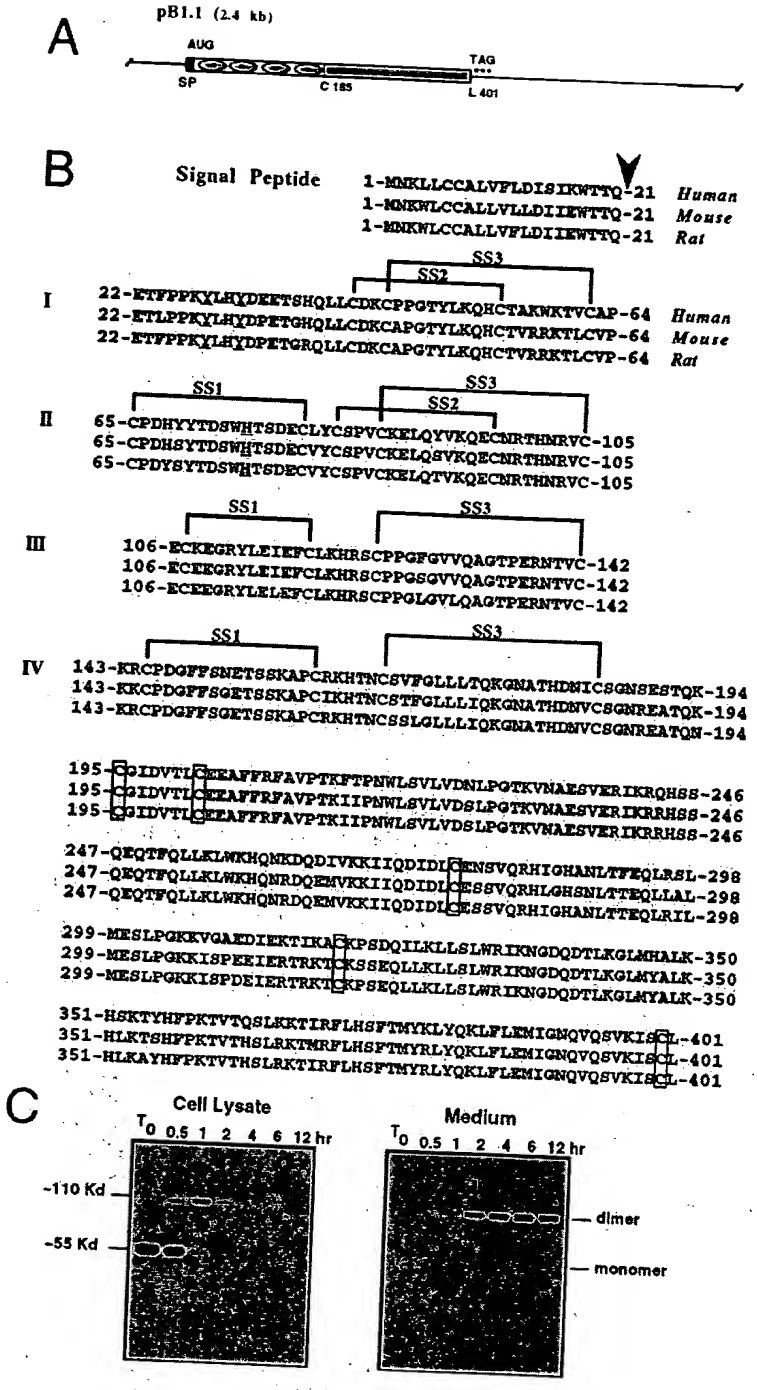


Figure 1. Osteoprotegerin Protein Structure and Secretion in Mammalian Cells

(A) Structure of the 2.4 kb rat intestinal pB1.1 clone indicating the position of the 401 aa open reading frame (bar). The closed box represents the 21-residue signal peptide, and shaded circles indicate the position of the 4 TNFR-like cysteine-rich domains.

(B) Alignment of human, mouse, and rat amino acid sequences. The location of the signal peptide cleavage site is indicated by a descending arrowhead. The four N-terminal TNFR-like cysteine-rich domains located between residues 22 and 194 are labeled I–IV. The predicted disulfide linkages (SS) for domains I–IV are indicated by bold lines. Either tyrosine 28 or 31 and histidine 75 (underlined) are predicted to form an ionic interaction. The cysteine residues located in the C-terminal half of OPG are boxed.

(C) Pulse-chase analysis of recombinant murine OPG produced in CHO cells. Cells were pulse-labeled for 30 min, then chased for the indicated time. Extracts of cells (left panel) and conditioned media (right panel) were immunoprecipitated, then analyzed by SDS–PAGE under nonreducing conditions. The relative mobility of the 55 kDa monomer and 110 kDa dimer are indicated.

(Figure 1A). This novel cDNA encodes a 401 amino acid (aa) polypeptide that has features of a secreted glycoprotein, such as a hydrophobic leader peptide, and 4 potential sites of N-linked glycosylation (Figure 1B). Although there were no existing matches in the database to the 401 aa reading frame, the predicted N-terminal half of the protein has a strong similarity to all members of the TNFR superfamily, most notably TNFR-2 and CD40. Unlike other known TNFR-like molecules, however, this protein contains no hydrophobic transmembrane-spanning sequence. The C-terminal half of OPG (aa 195–401) showed no homologies to any other known proteins nor contained any recognizable protein motifs. These data suggest that the rat *opg* cDNA encodes a novel secreted member of the TNFR family. Mouse and human *opg* cDNA clones isolated from normal kidney cDNA libraries also encode 401 aa proteins. The mouse and human proteins are ~85% and 94% identical to the predicted rat protein, respectively, indicating that the *opg* gene has been highly conserved throughout evolution (Figure 1B).

Pulse-chase labeling and immunoprecipitation of extracts obtained from OPG-transfected Chinese hamster ovary (CHO) cells show that OPG is a naturally secreted

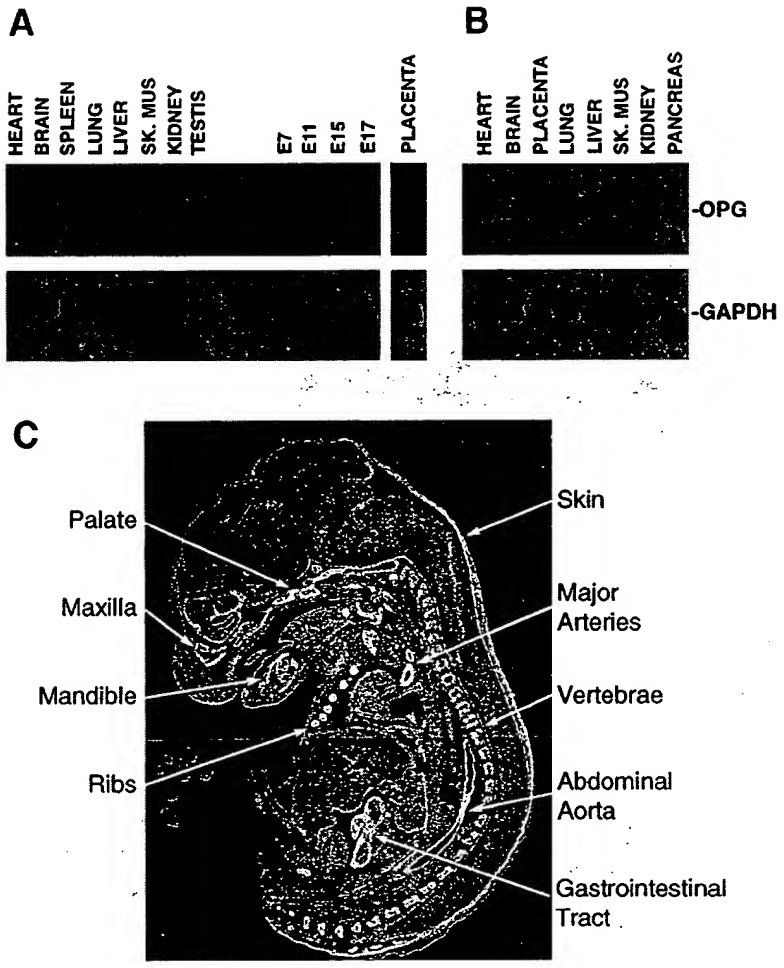


Figure 2. *opg* mRNA Expression in Tissues and during Mouse Embryogenesis

(A) Northern blot of poly(A)$^+$ mouse tissue or whole embryo RNA probed with a mouse *opg* cDNA probe (upper panel) or with a mouse *gapdh* cDNA probe (lower panel).

(B) Northern blot of poly(A)$^+$ human tissue RNA probed with a human *opg* cDNA probe (upper panel) or a mouse *gapdh* cDNA probe (lower panel). Both mouse and human OPG transcripts migrate as an approximately 3.0 kb band.

(C) In situ hybridization analysis of mouse *opg* mRNA expression in an E15 mouse embryo. Shown here is a saggital section of an E15 BDF1 mouse embryo that was hybridized overnight with [^{35}S]UTP antisense riboprobe for the detection of murine *opg* mRNA. Sense probe was used as a control and showed no signal (data not shown). Note the high expression of *opg* mRNA in cartilage rudiments of developing bones and in several major arteries, the gastrointestinal tract, and skin.

glycoprotein (Figure 1C). Initially, OPG is synthesized as an approximately 55 kDa monomer within the cell, is next converted to a disulfide-linked dimer of approximately 110 kDa, and is then secreted into the media (Figure 1C). While smaller amounts of monomeric OPG also are secreted, the predominant extracellular form of OPG is a disulfide-linked dimer. The monomeric and dimeric forms of OPG have been individually purified, and their N-terminal sequence has been determined (P. D., unpublished data). Both forms of OPG are cleaved during processing just before the glutamic acid residue at position 22 (Figure 1B), giving rise to a 380-residue mature protein. Under reducing conditions, OPG migrates as an approximately 55 kDa protein, larger than the 40 kDa predicted size of the mature polypeptide chain (data not shown). Upon treatment of purified OPG with N-glycanase, it migrates on reducing gels with an M_r of about 40 (M. K. and P. D., unpublished data), indicating that OPG is a glycoprotein. Together with the structural data described above, this suggests that OPG functions outside the cell as a secreted molecule capable of forming covalently bonded homodimers.

Patterns of OPG Expression in Mouse Tissues

opg transcripts of about 3.0 kb were detected by Northern blot hybridization in a number of murine tissues, including liver, lung, heart, and kidney (Figure 2A). *opg* mRNA is also expressed at high levels in the stomach, intestines, skin, and calvaria (data not shown). In human tissues, a transcript of similar size is detected at highest levels in the lung, heart, kidney, and placenta (Figure 2B), although there are detectable levels in various hematopoietic and immune organs (data not shown). During mouse embryonic development, *opg* transcripts are detected at high levels on day 7, whereas expression decreases at day 11 and then increases from day 15 to day 17 (Figure 2A). Since *opg* mRNA is expressed at relatively high levels in both mouse and human placenta, *opg* transcripts detected at day 7 may be due to the association of the placenta with the embryo at this developmental stage. Interestingly, there is a strikingly different pattern of OPG expression when comparing mouse and human tissues, most noticeably between brain, liver, and kidney. The reason for this is not clear, but it may reflect authentic differences in *opg* gene expression patterns between these two species. Alternatively, the levels of *opg* mRNA may physiologically vary, an aspect of this novel molecule that will need further study. Since OPG is a secreted protein, however, the site of its expression does not necessarily predict the site(s) at which it exerts its biological function.

Detection of *opg* mRNA transcripts by in situ hybridization in a day 15 mouse embryo is shown in Figure 2C. There is prominent expression of *opg* transcripts within the cartilagenous primordia of developing bone, most noticeably in the maxilla, mandible, the hyoid bone,

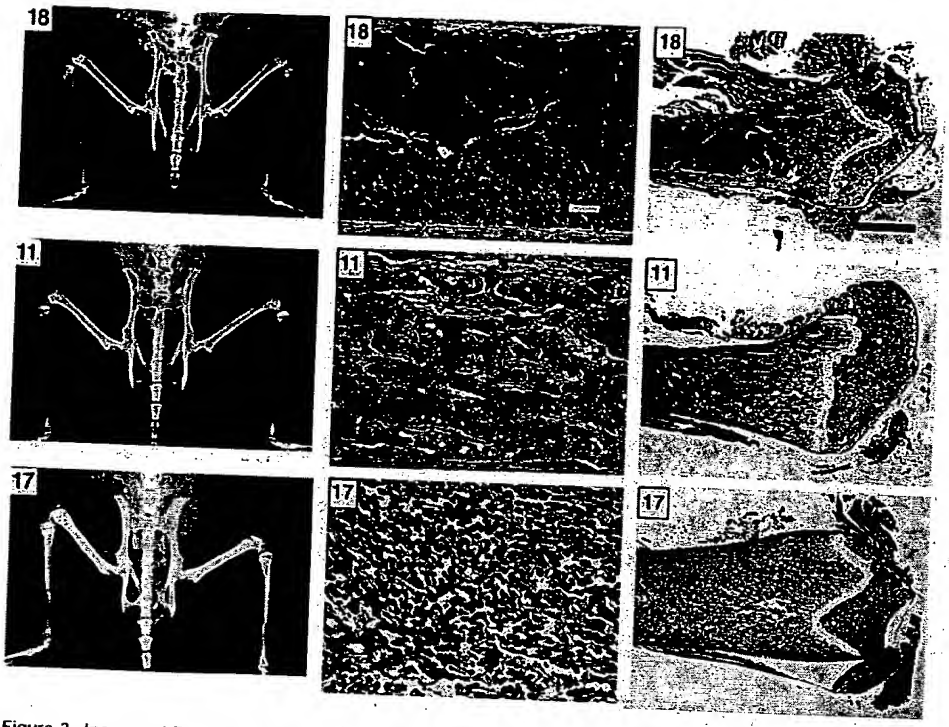


Figure 3. Increased Bone Density in *opg* Transgenic Mice

Two 10-week-old founder mice expressing relatively high (animal 17, bottom row) and low (animal 11, middle row) levels of *opg* transgene mRNA, and control littermate mouse (animal 18, top row) were subjected to X-ray (left column) or histologic (middle and right columns) analysis of their long bones, pelvis, and vertebrae. Increased bone density is seen in radiographs of transgenics 11 and 17, as are visible increases in bone by histological analysis. The lower power magnification (right column; bar = 1 mm) shows the distal femur and growth plate, and the higher power magnification (middle column; bar = 100 μm), shows the femoral midshaft. In these sections, bone stains deep blue or red, cartilage stains light blue, and marrow stains dark purple. Note the marbling effect reflecting the retention of cartilage remnants within trabeculae and the poorly defined cortex in the *opg* transgenics, particularly in animal 17 (lower middle and lower right panels, respectively). Increases in bone density are visible at birth in the high expressors and are detected with 100% penetrance in transgenic mice with elevated circulating levels of OPG.

the basiphenoid and basioccipital bones, the ribs, and the vertebrae. High expression was also detected in the brachiocephalic artery and ductus arteriosus, the left main bronchus, the abdominal aorta, and the midgut.

OPG Increases Bone Density In Vivo

To gain insights into the potential biological function for OPG, transgenic mice were generated that express the rat *opg* cDNA under the control of the human apolipoprotein E gene promoter and its associated liver-specific enhancer (Simonet et al., 1993). This vector has been previously used to generate mice containing high levels of secreted protein in their circulation (Simonet et al., 1994). Since OPG is a secreted receptor, any observable phenotype in mice overexpressing this protein should be the result of interactions with its cognate ligand, whether it is a soluble or a cell-surface protein. Five founder mice harboring the rat *opg* transgene were identified by Southern blot analysis of ear DNA samples and were analyzed at 8–10 weeks of age. Four of the five founders were found to express varying levels of transgene mRNA in their livers (data not shown). The rat OPG monomer and dimer were easily detectable in liver extracts and serum of high expressing transgenic mice but not control mice, indicating the transgene product was successfully delivered to the systemic circulation (W. S. S. and W. J. B., unpublished data).

The OPG-expressing animals and their normal littermates had no differences in external appearance, behavior, or bodyweight. Radiographs of intact animals showed a generalized increase in radiodensity of the long bones, vertebrae, and pelvis characteristic of osteopetrosis (Figure 3, left column). Despite this increase in radiodensity, there were no abnormalities in tooth eruption, which has been observed in other osteopetrotic mice (Yoshida et al., 1990; Soriano et al., 1991; Wang et al., 1992). Additionally, the relative size and shape of the bones in the expressors were not different from those of control mice. Upon gross dissection, splenomegaly was the only abnormality found in the transgene expressors. Analysis of organ weights indicated that only the spleens were increased (by ~38%) in the transgenic mice relative to controls. High expressors exhibited clear signs of osteopetrosis by radiography immediately following birth, with increasing severity throughout adolescence and adult life.

Histological analysis of bone sections from OPG expressors (Figure 3, founder animals 11 and 17) show severe osteopetrosis with the presence of trabeculae formed of bone-encased cartilage in the mid-diaphysis of the femur (Figure 3, middle column), a location normally

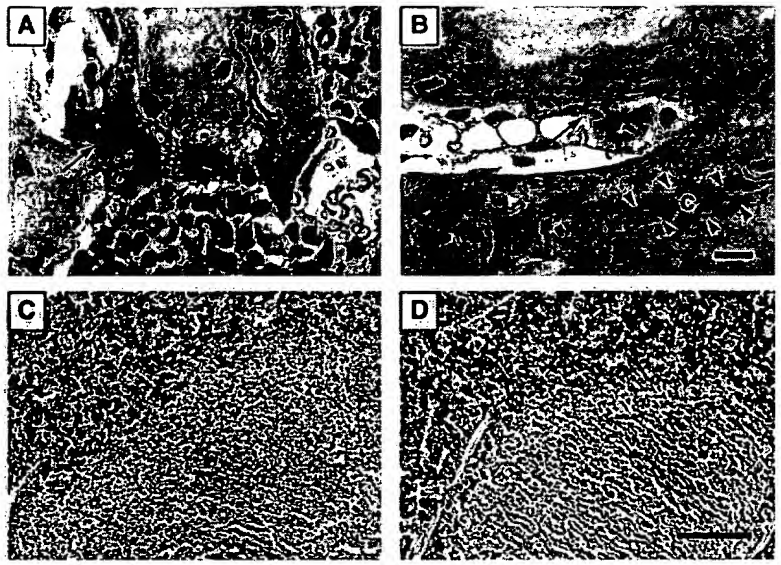


Figure 4. *opg* Transgenics Do Not Have a Defect in Monocyte–Macrophage Development but Appear to Have a Defect in Osteoclast Formation

Osteoclasts (arrows) in the distal femoral metaphysis were identified by TRAP cytochemistry. Many large multinucleated osteoclasts were present on the surface of metaphyseal trabecular bone in normal mice (A), while in the *opg* transgenic mice (B) only a few small TRAP-positive cells (possibly inactive osteoclasts) were seen in the same region. A cartilage remnant is marked "c," and its edge marked by arrowheads (bar = 10 μm). To confirm the presence of tissue macrophages, which share a common precursor with osteoclasts, immunohistochemistry was performed using anti-F480 antibodies, which recognize a cell-surface antigen on murine macrophages. The bottom panels are photomicrographs of spleens from either normal (C) or *opg* transgenic (D). Numerous F480-positive cells (brown staining) are detected in both spleens (bar = 100 μm). This is in marked contrast to what is observed in the osteopetrotic *Op* mice, which exhibit a defect in monocyte–macrophage development and a corresponding decrease in F480-positive cells in their spleens (Yoshida et al., 1990), suggesting that OPG is functioning to block osteoclast development at a more terminal step than the divergence of macrophage and osteoclast precursors.

filled by hematopoietic elements. The severity of the osteopetrosis correlated with the level of *opg* mRNA. In the highest expressors, a clearly defined cortex was not identifiable in sections of the femur (Figure 3, right column). Sections of vertebrae also show osteopetrotic changes, implying that the OPG-induced skeletal changes were systemic. Von Kossa staining of nondecalcified bone sections showed that the increased bone/cartilage matrix was mineralized (data not shown). Coincident with the loss of the marrow cavities, the spleens from the OPG expressors had an increased amount of red pulp, likely resulting from compensatory hematopoiesis owing to occlusion of marrow cavities with bone and cartilage. In addition to this, the highest OPG expressors had foci of extramedullary hematopoiesis within the liver (data not shown).

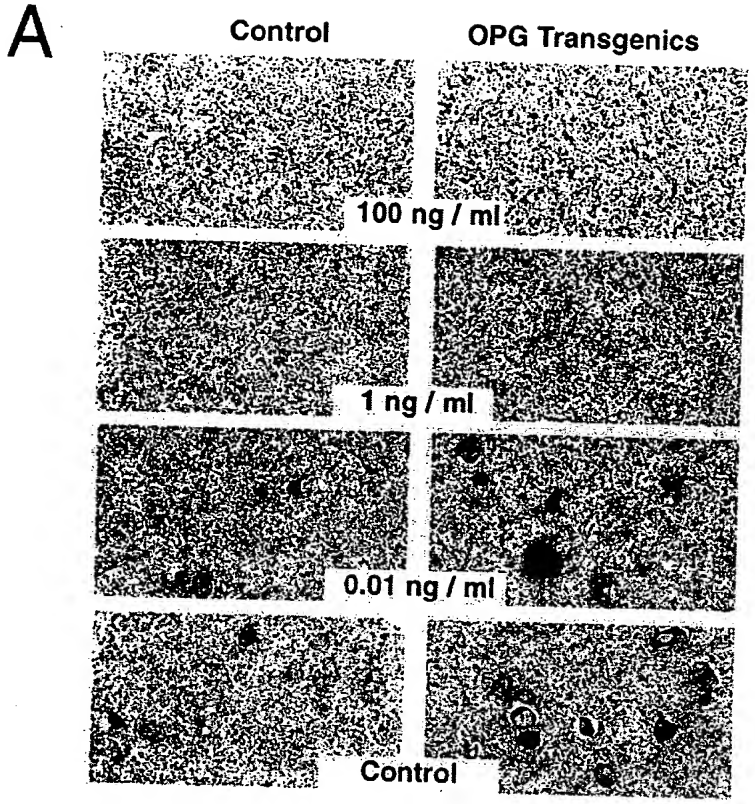
There were no other apparent gross pathologies in these mice, nor histologic abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreato-hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, heart and aorta, breast, skeletal muscle, or fat. These observations underscore the apparent skeletal selectivity of transgenically expressed *opg* activity.

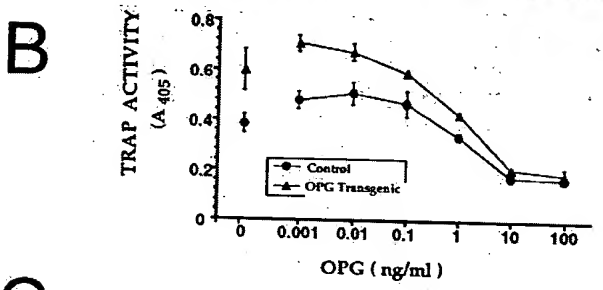
The observed increase in bone density seen in *opg* transgenic mice could be due either to increased osteoblast synthesis of bone matrix or, conversely, to decreases in osteoclast-mediated bone resorption. The analysis of bone sections suggests that the latter process was affected. Based on H and E and tartrate-resistant acid phosphatase (TRAP) stains, the predominant difference in the expressors was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs (Figures 4A and 4B). This was particularly evident in the most severely affected animals. Similar to the severity of the osteopetrosis observed histologically, the magnitude of osteoclast deficiency correlated with the relative level of hepatic *opg* transgene mRNA. Circulating levels of OPG in these founder mice were not determined. However, F_1 transgenic progeny with radiographic and histological bone changes similar to founder mice 17 and 11 (Figure 3) had circulating OPG levels of 358 ± 79 and 14.2 ± 3.6 ng/ml (mean ± SD), respectively. Penetrance of the osteopetrotic phenotype was found to be 100% in mice with serum OPG levels greater than 10 ng/ml and varied in severity proportional to the level of systemic OPG. In contrast, OPG levels in the normal littermates were less than 2 ng/ml. (L. B., unpublished data).

The residual marrow present as islands within the osteosclerotic bone of high expressors showed predominantly myeloid elements. Megakaryocytes also were present at normal levels. Immunohistochemical staining of tissues for F480, a cell-surface antigen expressed by osteoclast precursors of the monocyte–macrophage lineage (Austyn and Gordon, 1981), showed the presence of positive cells in the marrow spaces and flattened directly adjacent to trabecular bone surfaces (data not shown). All hematopoietic elements and F480-positive cells were present in red pulp of both control and OPG mice (Figures 4C and 4D). In contrast, F480-positive cells are virtually absent in *Op* mutant mice, which carry a defective *csf-1* gene (Yoshida et al., 1990). The presence of F480-positive cells in *opg* transgenic mice rules out a generalized decrease in osteoclast precursors as the basis for the effects of OPG and suggests that OPG might regulate the terminal stages of osteoclast differentiation.

Recombinant OPG Blocks Osteoclastogenesis In Vitro

To determine whether or not OPG acts to regulate osteoclast differentiation, its effects were tested in an in vitro





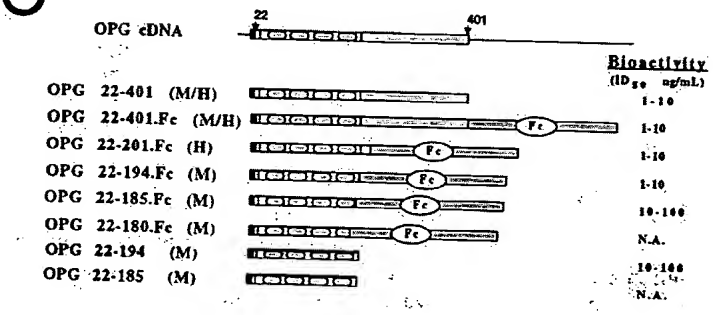


Figure 5. OPG Inhibits Osteoclastogenesis In Vitro

(A and B) Increasing concentrations of murine OPG [22–401]-Fc protein cause a dose-dependent decrease in the appearance of TRAP-positive osteoclasts (A) and TRAP activity (B) in osteoclast-forming cultures using splenocytes from either transgenic mice or their normal littermates (for assay details, see Experimental Procedures). Large multinucleated cells stained purple are mature osteoclasts. (B) shows the measurement of TRAP activity in treated cultures. Absorbance at 405 nm (A405) of lysed cultures plotted against OPG concentration indicates the conversion of p-nitrophenyl phosphate into p-nitophenol in the presence of sodium tartrate.

(C) Mapping the domain of OPG biological activity using the osteoclast-forming assay. Various constructs were used to make murine and human OPG proteins of varying lengths. The construct length (left) and its relative bioactivity (right) are indicated. Bioactivity is expressed as the range in protein concentration (ng/ml) rendering half-maximal activity (ID_{50}). OPG proteins that are not active are indicated (N.A.). Note that truncation of the molecule proximal to cysteine residue 185, when fused to human Fc, led to a complete loss of biological activity.

osteoclast-forming assay (Lacey et al., 1995). This culture system allows maturation of osteoclasts from hematopoietic precursors that express multiple differentiation markers of the osteoclast lineage, most notably TRAP. Recombinant murine OPG fused to the human immunoglobulin IgG_1 Fc domain (murine OPG [22–401]-Fc) was purified from CHO cell-conditioned media and subsequently tested in the osteoclast-forming assay. In the absence of exogenous OPG, spleen cells from both the OPG-expressing mice and controls contained osteoclast precursors (Figure 5A), which confirms that there is no intrinsic defect in osteoclast development in OPG mice. At 100 and 10 ng/ml of the murine OPG, osteoclast formation from spleen cells of both control and OPG mice was completely inhibited (Figure 5A). The levels of TRAP were also inhibited in the presence of OPG, with an ID_{50} of about 1 ng/ml (Figure 5B). The levels of TRAP activity in culture lysates appeared to correlate with the

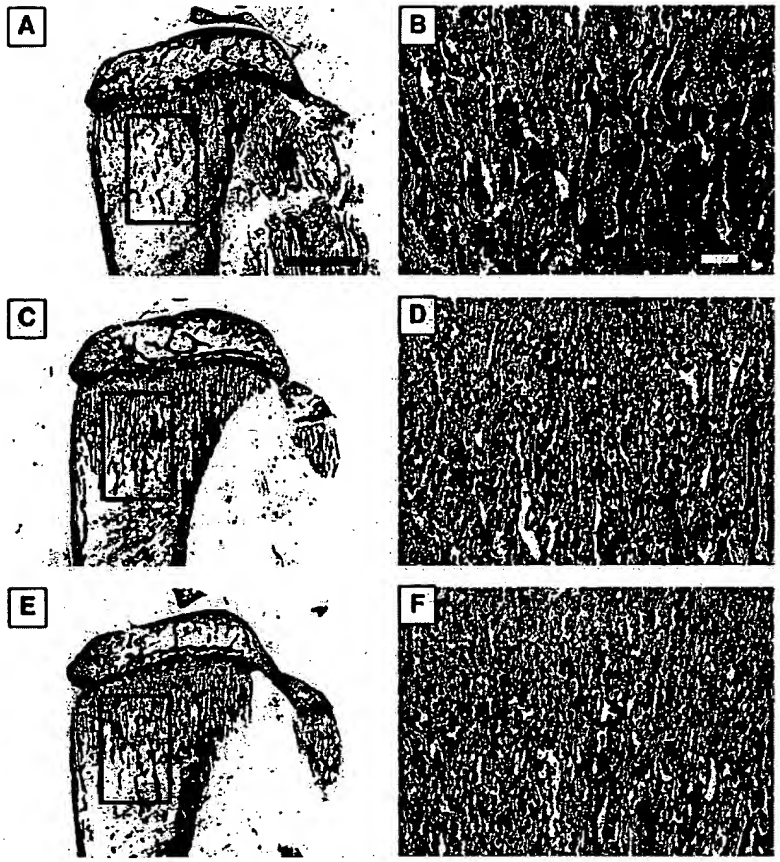


Figure 6. Recombinant OPG Administration Increases Bone Density in Normal Mice

Four-week-old male BDF1 mice (n = 5 per group) received subcutaneous injections of either vehicle (A and B), murine OPG [22–401]-Fc protein (10 mg/kg/day) (C and D), or the known antiresorptive pamidronate (5 mg/kg/day) (E and F) for seven days. The left panels are photomicrographs of Von Kossa-stained frozen sections of the proximal tibial metaphysis. Mineralized bone matrix is stained black in these micrographs, and shown is a similar visible increase in bone density in OPG-treated and pamidronate-treated mice relative to controls. Bone density was measured in the region highlighted (enclosed rectangles) and found to be increased in OPG and pamidronate-treated mice about 2- to 3-fold (see text). The right panels are photomicrographs of decalcified Masson's trichrome-stained sections of the distal femoral metaphysis of vehicle-treated (B), OPG-treated (D), or pamidronate-treated (F) mice. Development of a marbled appearance in the bone owing to cartilage retention in the OPG-treated (D) and pamidronate-treated (F) mice is similar to that seen in the high expressing founder 17 (see Figure 3). Bars in (A) and (B) indicate intervals of 1 mm and 100 μm, respectively.

relative number of osteoclasts seen by TRAP cytochemistry.

Additional forms of the human and murine OPG molecules were tested to determine portions of the protein required for biological activity (Figure 5C). Truncation of OPG N-terminal to cysteine 185 inactivates this molecule, presumably by disrupting the SS3 disulfide bond of the TNFR-like domain 4 (see Figure 1B). However, loss of the C-terminal portion of the molecule up to aa 194 did not affect activity. Thus, the N-terminal portion of OPG containing the TNFR-like domain is necessary and sufficient to inhibit osteoclastogenesis. All active forms of OPG inhibit osteoclastogenesis in a dose-dependent manner and possess half-maximal activities in the range observed for other potent cytokines in their respective in vitro assays (Figure 5C).

Recombinant OPG Increases Bone Density In Vivo

The transgenic model of OPG expression suggests that increases in bone and cartilage are due to elevated levels of circulating OPG. To prove this, OPG was administered to normal mice, and its effects on bone mass were measured and compared to the known antiresorptive bisphosphonate, pamidronate (Sietsema et al., 1989). Young rapidly growing mice (4 weeks of age) were subcutaneously injected daily with murine OPG [22–401]-Fc protein (10 mg/kg/day) or pamidronate (5 mg/kg/day). After 7 days, both the OPG and pamidronate recipients exhibited a marked increase in trabecular bone in the area of the tibial metaphysis relative to controls (Figure 6). A similar activity has been reported for this and other bisphosphonates (Sietsema et al., 1989). Based on histomorphometry of the proximal tibial metaphysis (Figures 6A, 6C, and 6E), the OPG-treated mice had an approximately 3-fold increase in trabecular bone compared to controls (31.1% ± 4.1% versus 12.0% ± 2.4%, respectively; mean ± SD, n = 5), which is highly significant ($p < 0.0001$). Pamidronate treatments also significantly increased trabecular bone volume (22.9% ± 0.6% over controls, $p < 0.001$). Administration of recombinant OPG or pamidronate also increased bone density in the metaphysis of the distal femur (Figures 6B, 6D, and 6F) and produced a pattern of cartilage retention within the bone trabeculae similar to that seen in transgenic founder 17 (Figure 3). In the OPG recipients, the serum level of OPG 24 hr after the last injection was 320 ± 176 ng/ml, a level similar to the steady-state levels in transgenic mice with a severe phenotype. Collectively, these data indicate that OPG acts as an antiresorptive agent that negatively regulates osteoclast maturation and can lead to the excess accumulation of newly synthesized bone and cartilage in vivo.

Recombinant OPG Protects Rats against Ovariectomy-Associated Bone Loss

A key test of the biological activity of OPG is to demonstrate that it can protect against pathological decreases of bone volume associated with loss of estrogen in ovariectomized rats. The ovariectomized rat is a commonly used animal model of human postmenopausal bone loss that is mediated by increased osteoclast activity (Christiansen et al., 1980; Kalu et al., 1989). Fisher rats, aged

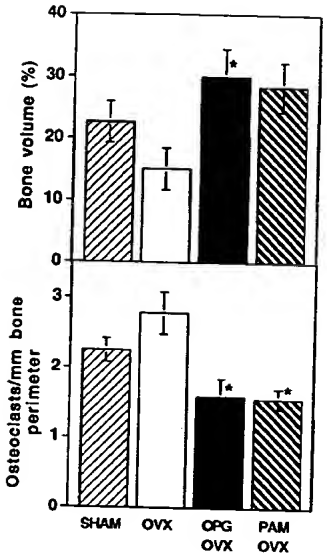


Figure 7. Recombinant OPG Blocks Ovariectomy-Induced Bone Loss in Fisher Rats

Twelve-week-old Fisher rats were ovariectomized and treated for 14 days with vehicle (OVX), murine OPG [22–401]-Fc protein (5 mg/kg/day) (OVX OPG), or pamidronate (5mg/kg/day) (OVX PAM). Sham-operated animals were treated with vehicle (SHAM). Bone volume in the proximal tibial metaphysis was increased in OPG OVX rats relative to OVX (upper panel, asterisk means different to ovariectomized animals, $p < 0.05$), and a similar trend is apparent in the SHAM and PAM OVX groups. Osteoclast numbers in the distal femoral metaphysis were decreased in the OPG OVX and PAM OVX relative to OVX (lower panel, asterisk means different to ovariectomized animals, $p < 0.05$).

12 weeks, were ovariectomized and after 4 days treated for 2 weeks with recombinant murine OPG [22–401]-Fc protein (5 mg/kg/day) or pamidronate (5 mg/kg/day). OPG-treated rats exhibited an increase in bone volume and a decrease in osteoclast numbers relative to controls (Figure 7). The known antiresorptive, i.e., pamidronate, had effects similar to OPG and those reported for other bisphosphonates (Wronski et al., 1989). These data show that OPG can act as a protective agent against inappropriately high rates of osteoclast-mediated bone resorption, in addition to its effects in normal animals.

Discussion

OPG is a novel secreted member of the TNFR superfamily, which inhibits osteoclast maturation and protects bone from both normal osteoclast remodeling and ovariectomy-associated bone loss. This protein contains two characterized domains: the N-terminal half, which harbors four tandem cysteine-rich TNFR motifs, and the C-terminal half, which is unrelated to any known protein sequences but appears to function in the association of OPG monomers as they are processed within the secretory pathway (W. J. B. and A. C., unpublished data). The TNFR superfamily consists primarily of transmembrane proteins that elicit signal transduction in a variety of cells and are known to mediate diverse biological responses, including cytotoxicity and apoptosis, and cell survival, proliferation, and differentiation (Smith et al., 1994). Soluble forms of these receptors have been identified and are generated either by cleavage from the cell membrane or as secreted molecules encoded by alternatively spliced mRNAs (Kohno et al., 1990; Hughes and Crispe, 1995). The soluble forms of these receptors are likely to capture and bind their cognate ligands, thereby preventing the ligands from activating their cellular targets. Several members of the Pox family of viruses express soluble TNF receptors, which aid in neutralizing the antiviral effects of TNFα (Smith et al., 1994; Schreiber et al., 1996). Interestingly, the only form of OPG that we have isolated is a secreted protein. Based on our analysis, we have not detected any qualitatively altered transcripts capable of encoding a transmembrane form of the protein.

Systemic delivery of OPG via the expression of rat or murine *opg* transgenes in mice results in severe yet nonlethal osteopetrosis. The osteopetrotic phenotype caused by OPG overexpression differs significantly from those observed in other mouse osteopetrotic models, whether generated by the disruption of specific genes (*src* or *fos* gene knockouts) or in naturally occurring mouse mutants (Marks, 1989; Yoshida et al., 1990). Osteopetrosis is often lethal in those models, and bone formation, as well as tooth eruption, are abnormal. *opg* transgenic mice are, in contrast, of normal size, have no apparent defects in tooth eruption, and have normally shaped bones. Histologically, the *opg* transgenic mice have a marked reduction in trabecular osteoclasts but no deficiency of osteoclast precursors, suggesting a defect in the later stages of osteoclast differentiation. *opg* transgenic mice do not appear to have any defects in the normal development of osteoclast progenitor cells, since osteoclast-like cells develop readily from in vitro cultures of *opg* transgenic spleen cells. Taken together, these findings suggest that OPG, and perhaps its ligand(s) or receptor(s), naturally serve to regulate bone density by modulating osteoclast differentiation from hematopoietic precursors.

Recombinant OPG protein inhibits osteoclast differentiation in a dose-dependent manner in an in vitro osteoclast-forming assay. The N-terminal 185 amino acids of OPG are required for activity, and truncations that disrupt the predicted SS3 bond of domain 4 eliminate activity. This suggests that the TNFR-related portion of OPG, which resembles the ligand-binding domain of other related receptors, is sufficient for arresting osteoclast maturation in vitro. One hypothesis for this activity is that OPG is neutralizing a TNF-related protein that normally acts as an osteoclast maturation factor. There are no known members of the TNF family that appear to possess this activity, although TNFα can induce bone resorption (Bertolini et al., 1986) and increase osteoclast formation in vitro (Thomson et al., 1987). It is noteworthy that TNF-binding protein, which does neutralize TNFα activity, fails to inhibit osteoclastogenesis in vitro and does not induce the accumulation of bone and cartilage in normal mice (D. L. L. and H.-L. T., unpublished data). Alternatively, OPG may bind to a TNF-related protein that acts as a receptor, a plausible theory since a majority of TNF family members are transmembrane proteins.

Several lines of evidence indicate that OPG may be

a key determinant regulating bone metabolism. First, overexpression in transgenic mice at a site distant to the bone results in a dramatic increase in bone density and inhibition of osteoclast maturation. The severity of the phenotype in the transgenics correlates with the levels of RNA expression in the liver and with circulating levels of protein. Second, OPG specifically inhibits osteoclastogenesis in vitro. Third, systemic administration of OPG produces an increase in bone density in the tibial metaphysis and blocks the loss of bone induced by ovariectomy. Fourth, in situ hybridization data indicate that the mouse gene is expressed at high levels in the cartilaginous primordia of bones throughout the fetus. Lastly, the human gene is localized to chromosome 8 q23–24, a region closely linked to the gene involved in hereditary multiple exocytoses (HME), a skeletal disorder resulting in bone malformation and, in some instances, chondrosarcoma (Ahn et al., 1995). This same locus harbors the gene for the bone morphogen BMP-1 (Tabas et al., 1991), a member of the TGFβ superfamily that has potent bone-forming activity in vivo, even in soft tissue sites away from bone. This raises the possibility that this region of chromosome 8 may harbor a gene cluster involved in the regulation of bone development and metabolism.

In summary, a novel member of the TNF receptor family has been identified, and its biological function deduced. Unlike other members of this family, Osteoprotegerin appears to function as a secreted protein. In addition, a novel gain-of-function model for nonlethal osteopetrosis has been generated by transgenic overexpression of OPG in mice. The phenotype of this model differs significantly from models generated by the targeted mutagenesis of the *src* and *fos* genes, and from naturally occurring mutant mouse strains, such as the *Op* mouse (CSF-1 disruption). Our analysis suggests that a step in osteoclast differentiation is impacted by OPG in vitro. Therefore, we propose that OPG is a secreted regulator of bone density that can act locally and systemically by negatively regulating osteoclast maturation. Our initial findings imply a utility for OPG in the treatment of osteopenic disorders that are characterized by excessive osteoclast activity, such as primary osteoporosis, Paget's disease of the bone, hypercalcemia of malignancy, and osteolytic metastases.

Experimental Procedures

Computational Biology

A cDNA library was constructed using mRNA isolated from rat embryonic d20 intestine for EST analysis (Adams et al., 1991). The resulting 5′ nucleotide sequence obtained from individual clones was translated and compared with the existing database of known protein sequences using a modified version of the FASTA program (Pearson, 1990). Analyses for the presence of specific protein motifs were carried out using a modified sequence profile (Lüthy et al., 1994).

A full-length *opg* cDNA clone containing a 2.4 kb insert (pB1.1) was isolated from a fetal rat intestine cDNA library by colony hybridization and sequenced. The mouse and human homologs of the rat *opg* cDNA were isolated from either mouse or human normal kidney cDNA libraries (Clontech, Palo Alto, CA) by PCR amplification and colony hybridization, then sequenced. A human DNA clone was obtained from a genomic P1 library (Genome Systems Inc., St. Louis, MO) by plaque hybridization with a labeled human *opg* cDNA probe and was used in fluorescence in situ hybridization (FISH) analysis to determine its chromosomal localization in metaphase chromosomes as described (Heng et al., 1992).

Expression and Analysis of Recombinant OPG Protein

Mouse and human OPG-Fc fusion protein vectors were constructed by PCR as previously described (Byrn et al., 1990) with minor modifications. The full-length *opg* reading frame was PCR amplified, then linked to glutamic acid residue 211 of the human IgG_1 Fc region through an artificial NotI adapter, creating the following junction sequence: KISCLAAAEPKSCD.

Variants of the human and mouse full-length OPG-Fc fusion proteins were constructed by PCR amplifying the OPG regions encoding residues 1–201 (human), 1–194 (mouse), 1–185 (mouse), and 1–180 (mouse), then fused to Fc as described above. The OPG-Fc chimeric sequences were then cloned into the plasmid vector pCEP4 (Invitrogen, San Diego, CA). The parent pCEP4 and pCEP4 OPG-Fc vectors were lipofected into 293-EBNA-1 cells (Invitrogen, San Diego, CA) and selected in 100 μg/ml hygromycin using the manufacturer's recommended methods. Serum-free media was produced, and the OPG-Fc fusion proteins were purified by protein A/G-affinity chromatography (Ey et al., 1978). The mouse and human *opg* cDNAs were also cloned into the eukaryotic expression vector pDSRα and expressed in stably transformed CHO cell lines as previously described (DeClerk et al., 1991). The purification of native recombinant OPG from CHO cell-conditioned media will be described in another publication (M. K. and E. D., unpublished data).

Rabbit polyclonal antibodies were raised to purified murine OPG [22–401]-Fc fusion protein by subcutaneous injection of antigen emulsified in adjuvant, then affinity purified. A CHO cell line overexpressing the murine OPG protein was metabolically labeled and immunoprecipitated as previously described (Boyle et al., 1991). In brief, subconfluent cultures were pulse-labeled with approximately 1.0 mCi/ml of [^{35}S]-Translabel (ICN, Irvine, CA) for 30 min, then chased with complete media for the indicated time interval. The conditioned media was removed and made 1% in NP-40 (v/v), and the cell monolayers were lysed with RIPA buffer. Both the media and cell lysates were clarified, then immunoprecipitated. Western blots of conditioned media and mouse serum samples were performed as previously described (Kamps and Sefton, 1988). The blots were probed with anti-OPG antibodies (0.1 μg/ml), and immune complexes detected by enhanced chemiluminescence (Amersham) after exposure to Kodak X-ray film.

mRNA Analysis

Northern blots of either total or poly(A)$^+$ mRNA were prepared as described (Jähner and Hunter, 1991) or purchased from a commercial source (MTN's, Clonetech, Palo Alto, CA). All blots were probed under stringent conditions with a ^{32}P-dCTP-labeled rat, mouse, or human *opg* DNA probe as indicated. These blots were subsequently probed with the mouse *gapdh* cDNA to verify the integrity of the blotted mRNA. Northern blot analysis was also performed on founder liver tissue RNA to assess the expression of the rat *opg* transgene.

In situ hybridization was performed on mouse embryos fixed in 4% paraformaldehyde. Antisense and sense RNA probes to murine OPG (bp 803–1000) were transcribed using viral RNA polymerases in the presence of [^{35}S]UTP from a PCR-generated template containing the T7 promoter. Embryo sections were hybridized with the appropriate probe, then covered with emulsion and exposed 2–3 weeks at 15°C. The slides were counter stained with methyl green prior to photography.

Generation of *opg* Transgenic Mice

The coding region for either the rat or murine *opg* cDNA was subcloned free of mutations into an expression vector placing it under the control of the human ApoE promoter and liver-specific enhancer element (Simonet et al., 1994). For microinjection, the ApoE-OPG plasmid was purified, and the transgene insert isolated. Single-cell embryos from BDF1 mice were injected essentially as described (Brinster et al., 1985) and cultured overnight in a CO_2 incubator. Fifteen to twenty 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice. Transgenic offspring were

identified by screening for the *ApoE-opg* transgene in DNA prepared from ear biopsies as described (Simonet et al., 1994).

Necroscopy and Pathological Analysis

At 8–10 weeks of age, 5 transgenic founder and 5 control animals were necropsied. Radiography was performed prior to the gross dissection. Bone tissue was decalcified using a formic acid solution, and all sections were stained with H and E. In addition, staining with Gomori's reticulin and Masson's trichrome was performed on certain tissues. To assess the status of matrix mineralization, frozen sections of nondecalcified bone were stained using the Von Kossa method. Using this method, the mineralized matrix is black, while other tissues stain red. Enzyme histochemistry was performed to determine the expression of TRAP. Immunohistochemistry for the monocyte–macrophage F480 surface antigen was also performed on formalin-fixed paraffin-embedded 4 μm sections using the rat monoclonal anti-mouse F480 antibody (Harlan, Indianapolis, IN). The antibody was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase-conjugated strepavidin (BioGenex, San Ramon, CA), using DAB as the chromagen (BioTek, Santa Barbara, CA). Immunohistochemistry sections were counterstained with hematoxylin.

In Vitro Osteoclast-Forming Assay

A cell-culture system allowing osteoclast development from hematopoietic precursor cells was used to analyze the effects of recombinant OPG (Udagawa et al., 1989; Lacey et al., 1995). Spleen cells were cultured in vitro overnight in alpha MEM containing 10% heat-inactivated fetal bovine serum supplemented with 500 U/ml CSF-1. After this incubation, the nonadherent cells were collected, subjected to gradient purification, and then cocultured with the bone marrow stromal cell line ST2 at a ratio of 1×10^5 ST2 cells to 1×10^6 nonadherent spleen cells/ml of media supplemented with dexamethasone (100 nM) and 1,25 dihydroxyvitamin D_3 (10 nM). Prostaglandin E_2 (250 nM) was added to some cultures to enhance osteoclast formation (Lacey et al., 1995). Cocultures were incubated for 8–10 days. New media containing fresh supplements was added every 3–4 days. Osteoclast formation was measured by quantitating the presence of TRAP using either cytochemical staining or by a TRAP-solution assay (Lacey et al., 1995). In the TRAP-solution assay, enzyme activity/well was measured by the conversion of p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate and was expressed as optical density at 405 nm. To some cultures, recombinant OPG proteins were added at concentrations ranging from 0.001 ng/ml to 100 ng/ml during the coculture of nonadherent cells with ST2 cells. The half-maximal inhibitory dose (ng/ml) for OPG recombinant proteins (ID_{50}) was obtained semiquantitatively by extrapolating from the dose-response curves generated from OPG-treated cultures.

In Vivo Response to Recombinant OPG

Male BDF1 mice (Charles River, Wilmington, MA) aged 4 weeks were injected daily subcutaneously with recombinant murine OPG [22–401]-Fc fusion protein (10 mg/kg in phosphate-buffered saline), 5 mg/kg pamidronate ("Aredia," Ciba-Geigy, Tarrytown, NY), or carrier alone for 7 days (5 mice/group). On the day following the last injection, the mice were killed, blood was taken for the determination of circulating OPG levels, and the right tibia and femur were removed. The tibiae were frozen without decalcification, and longitudinal frozen sections cut through the midregion of the proximal metaphysis, using the fibula junction as a landmark. The sections were stained by Von Kossa stain to demonstrate mineralized bone and cartilage. Bone density was determined in a region 1×1.5 mm midway between the cortices adjacent to the edge of the growth plate by automated image analysis (Metamorph, Universal Imaging Systems, West Chester, PA). The femurs were decalcified and processed as described above.

Ovariectomized (n = 22) or sham-operated (n = 10) 12-week-old Fisher rats (Charles River, Wilmington, MA) were treated for 14 days with subcutaneous recombinant murine OPG [22–401]-Fc fusion protein in phosphate-buffered saline (5 mg/kg/day) or pamidronate (5 mg/kg/day), n = 6 per group. Sham-operated and 10 ovariectomized rats were treated with vehicle. The day following the last injection, rats were euthanized, and the right tibia and femur removed. Bone density was determined in frozen sections of the distal tibial metaphysis as described above. The femur was decalcified in formic acid, dehydrated, mounted, and stained with hematoxylin and eosin. Osteoclast numbers were determined in a region 1×0.6 mm proximal to the growth plate in the distal femoral metaphysis. Osteoclasts were identified microscopically based upon morphology and enumerated relative to bone perimeter with the aid of a digitizing platen and camera lucida. Measurements were analysed using Osteomeasure software (Osteometrics, Atlanta, GA). Statistical significance of results was evaluated by Dunnett's test to correct for multiple comparisons (JMP Statistical Software, SAS Institute, Cary, NC).

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Soluble and Cell Surface Receptors for Tumor Necrosis Factor

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Summary

Tumor necrosis factor (TNF) initiates its multiple effects on cell function by binding at a high affinity to specific cell surface receptors. Two different molecular species of these receptors, which are expressed differentially in different cells, have been identified. The cDNAs of both receptors have recently been cloned. Antibodies to one of these receptor species (the p55, type I receptor) can trigger a variety of TNF like effects by cross-linking of the receptor molecules. Thus, it is not TNF itself but its receptors that provide the signal for the response to this cytokine. The intracellular domains of the two receptors differ in structure, suggesting that they mediate different activities. Their extracellular domains, however, are structurally related. Both contain cysteine-rich repeats which are homologous to repeated structures found in the extracellular domains of the nerve growth factor receptor and the CDw40 protein. Truncated soluble forms of the two receptors, corresponding to these cysteine-rich repeated structures, have been detected in human urine and were later found to be present also in the serum. The serum levels of those soluble TNF receptors increase dramatically in certain pathological situations. Release of the soluble receptors from the cells seems to occur by proteolytic cleavage of the cell surface forms and appears to be a way of down-regulating the cell response to TNF. Because of their ability to bind TNF, the soluble receptors exert an inhibitory effect on TNF function, and may thus act as physiological attenuators of its activity.

Tumor necrosis factor (TNF), a polypeptide cytokine, produced primarily by mononuclear phagocytes, exerts effects on cell function which are involved in immune defense against a variety of different pathogenic agents. In certain diseases, however, TNF may itself become pathogenic. It is this later function of TNF that has attracted

so much attention, recently, to this cytokine (Beutler and Cerami, 1988; Old, 1988).

Although its existence has already been known for quite some time (Granger and Kolb, 1968; Ruddle and Waksman, 1968; Carswell et al., 1975), it is only a few years since TNF was isolated (Aggarwal et al., 1985; Haranaka et al., 1985; Beutler et al., 1985a; Hahn et al., 1985). Information on the molecular basis for its function is therefore still limited. Shortly after its isolation it was shown in a number of laboratories that TNF binds with high affinity to specific receptors, which are expressed by a variety of cells (Baglioni et al., 1985; Beutler et al., 1985a; Kull et al., 1985; Tsujimoto et al., 1985; Aggarwal et al., 1986; Israel et al., 1986). On the basis of this finding it could already be surmised that TNF was not, as previously suspected, a "killer" molecule having an intrinsic toxic activity, but a cytokine, and that as in the case of other cytokines, it is not TNF itself but its receptors that provide the signal for the cellular response. It took several more years, however, for the receptors to be isolated, which made it possible to obtain formal evidence for this notion and to approach the study of the intracellular signals triggered by the receptors. As in the purification of TNF itself, the isolation of its receptors was hampered by the low abundancy of these molecules. Although these receptors are present in almost all cells, their amounts are very low, usually in the range of a few thousands of molecules per cell. Even more limiting, however, were difficulties arising out of the fact that these proteins are membrane-associated. Because of certain characteristics of the TNF molecule itself, the usual methods employed in the isolation of membranous proteins from detergent extracts of cells were not easily applicable here. Since these receptors exist in nature also in soluble forms, it is not surprising that in more than one laboratory the isolation of these soluble forms was achieved first, paving the way to the isolation of the membranous receptors.

Identification of Two TNF-Binding Proteins Present in Human Urine as Soluble Forms of Two Molecular Species of the Cell Surface TNF Receptors

A number of studies have shown that proteins of human urine exert a marked inhibitory effect on the *in vitro* function of TNF. Attempts

to identify the proteins having this activity led to the successive isolation of two minor urinary proteins with TNF binding activity. These TNF-binding proteins (which we termed TBPI and TBPII) are similar in size (about 30,000) yet distinct immunologically (Engelmann et al., 1989, 1990a,b; Olsson et al., 1989; Seckinger et al., 1989).

Antibodies against both proteins inhibited the binding of TNF to cells. However their respective inhibitory capacities varied with the kind of cell examined (Engelmann et al., 1990a). This finding suggested that TBPI and TBPII cross-react immunologically with cell surface TNF receptors (TNF-Rs) of two different molecular species, which are expressed differentially in different cells. Indeed, further examination revealed that antibodies against TBPI immunoprecipitate a TNF-R of Mr 550000 (the type I or p55 receptor) from cell extracts, while antibodies to TBPII immunoprecipitate a receptor of Mr around 75000 (the type II or p75 receptor) (Engelmann et al., 1990b). The structural relationships between the two soluble TNF-binding proteins and their respective receptors were fully clarified after cloning of the cDNAs for the receptors (Loetscher et al., 1990; Schall et al., 1990; Nophar et al., 1990; Smith et al., 1990; Heller et al., 1990; Kohno et al., 1990). In some of the studied that led to the cloning, it was the knowledge of this structural relationship and of the structure of the soluble proteins which provided the basis for the cloning approach. Examination of the amino acid sequences in the receptors, disclosed by the cloning of their cDNAs, revealed that they contain sequences found in the soluble proteins. Recently we determined the full sequence of amino acids in one of the two TNF-binding proteins (TBPI) and found it to be completely matched by the sequence of amino acids in the extracellular domain of the immunologically cross reacting cell surface receptor (the type I, p55 receptor). Although the extracellular domains of the two TNF-Rs have non-identical amino acid sequences, as would be expected from their failure to cross-react immunologically, their structure is highly homologous. Both contain conserved cysteine-rich repeat structures which extend over almost their entire length. The extracellular domains of two other known receptors – the receptor for the nerve growth factor and the B-cell-activating antigen, CDw40 – also contain this conserved repetitive structure.

The cysteine-rich repeat structures extend over almost the whole length of the soluble urine-derived TNF binding proteins (Nophar et al., 1990).

Use of Antibodies Against the Extracellular Domains of the TNF-Rs to Explore Their Mode of Action

A number of receptor-mediated effects can be mimicked by cross-linking of the relevant receptors. Similarly, effects which are characteristic of TNF could be induced in cells by antibodies against the extracellular domain of the type I TNF-R (Engelmann et al., 1990b; Espevik et al., 1990). This mimetic effect of the antibodies was not due to their ability to present to cells a molecular structure identical with that of TNF. A comparative study of the ability of various antibodies against the receptor to mimic the activity of TNF indicated that this activity is due merely to their ability to cross-link the receptors (Engelmann et al., 1990b). This finding demonstrated conclusively that it is not TNF itself but its receptors that provide the signal for the cellular response. It also raised the possibility that aggregation of the TNF-Rs occurs also upon their activation by TNF, a notion which seems further supported by the fact that the TNF molecule itself is an oligomer and loses bioactivity upon dissociation to its monomeric form.

There is still no information on the nature of the biochemical signals which the TNF-Rs provide. Rapidly induced phosphorylation of certain proteins, such as HSP27, observed in cells treated with TNF, may provide a clue to the identity of such signals. Although these effects can also be triggered by cross-linking with the type I TNF-R molecules (Nophar et al., 1990), the sequence of amino acids in the intracellular domains of the two TNF-Rs indicate that neither of the two receptor species possesses intrinsic kinase activity, suggesting the involvement of additional cellular components, perhaps receptor associated proteins, in providing the signals. Since there is no apparent structural similarity between the intracellular domains of the two species of TNF-R, it seems likely that there are differences in the signals which they provide as well as in their effects. Conceivably, the effect of the two receptors are cell specific, to the extent that the receptors themselves are.

Physiological Significance of the Soluble Forms of the TNF-Rs

Does the interaction of TNF with the soluble TNF-Rs significantly affect the *in vivo* function of this cytokine as it does *in vitro*? Evaluation of the physiological significance of an extracellular factor can be achieved only by studies carried out at the level of the whole organism, and therefore presents far greater difficulties than an evaluation of the function of a cell-associated component such as the cell surface TNF-Rs. Our present notions of the *in vivo* function of the soluble TNF-Rs can be based only on indirect information, such as knowledge of the kinds of situations in which the formation of these proteins occur and the nature of the mechanisms involved in this process.

ELISA tests for the determination of the serum levels of the two soluble TNF-Rs have revealed that both proteins are present in normal sera, though in low amounts, and that their serum levels increase dramatically in certain pathological states. The range of diseases in which such an increase occurs is very wide and includes numerous autoimmune diseases, infectious diseases and other inflammatory processes. Interestingly, both types of soluble receptors are increased also in the sera of patients with solid tumors or hematological malignancies. The incidence of such increase in cancer patients is rather high, exceeding even the incidence of increase of the carcinoembryonic antigen, one of the most widely used diagnostic parameters for cancer (Aderka et al., 1991). At least part of this increase may reflect shedding of the TNF-Rs by the tumor cells themselves. Indeed, we found continuous production of the soluble TNF-Rs in culture by cells of various tumor-derived lines. In normal cells, however, formation of the soluble TNF-Rs seems to be largely dependent on stimulation of the cells. The rapidity with which this process occurs in response to certain stimuli, as well as the absence of any detectable amounts of soluble TNF-Rs within cells, suggests that it reflects cleavage of the existing cell surface receptors, not *de novo* formation of the soluble receptors, by a mechanism independent of the formation of the membranous forms (Porteu and Nathan, 1990, Lantz et al., 1990). Consistently, it was found that in CHO cells transfected with cDNA for the type I TNF-R, the cDNA encodes both the cell surface and the soluble forms of the receptor

(Nophar et al., 1990). Activation of such a process of cleavage may result in a significant decrease in the amounts of cell surface TNF-Rs, and may thus represent a mode of down regulation of the response to TNF. In some of the pathological situations in which TNF may cause harm this process seems to occur at an increased rate, as reflected in significantly increased levels of soluble TNF-Rs in the sera of patients with those diseases. The concentrations of soluble receptors obtained in such cases are high enough to exert an inhibitory effect on TNF function *in vitro*, (unpublished data). It remains to be determined whether such inhibition is a significant factor in the *in vivo* response to TNF in these cases, and whether it can be further augmented by injecting patients with a higher dose of the soluble receptors.

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Characterization of Structural Domains of Human Osteoclastogenesis Inhibitory Factor*

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Osteoclastogenesis inhibitory factor (OCIF) is a heparin-binding secretory glycoprotein that belongs to the tumor necrosis factor receptor (TNFR) family. OCIF is present both as a ~60-kDa monomer and a disulfide-linked homodimer. We attempted to characterize the seven structural domains of OCIF by determining the capabilities of various OCIF mutants to inhibit osteoclastogenesis, to interact with heparin, and to form dimers. We also examined a potential of domains 5 and 6, death domain homologous regions (DDHs), for inducing cell death by expressing OCIF/Fas fusion proteins. Our results show that: (i) the N-terminal portion of OCIF containing domains 1–4, which have structural similarity to the extracellular domains of the TNFR family proteins, is sufficient to inhibit osteoclastogenesis; (ii) a heparin-binding site is located in domain 7, and affinity for heparin does not correlate with the inhibitory activity; (iii) Cys-400 in domain 7 is the residue responsible for dimer formation; and (iv) the C-terminal portion containing domains 5 and 6, DDHs, has a high potential for mediating a cytotoxic signal when it is expressed in cells as an OCIF/Fas fusion protein in which the transmembrane region of Fas is inserted in front of DDHs.

In the vertebrate, homeostasis and remodeling of bone are by strictly controlled by mostly unrevealed mechanisms. Much effort has been made to clarify the mechanisms, and several protein factors were found to participate in bone homeostasis (1–3). Recently, we have isolated one such factor termed osteoclastogenesis inhibitory factor (OCIF)[1] from the conditioned medium of human embryonic lung fibroblasts, IMR-90 (4). Both a ~60-kDa monomer and a disulfide-linked homodimer are present in the conditioned medium, and the two forms have similar specific activity in inhibition of osteoclast formation *in vitro* (4). However, the mechanism by which OCIF inhibits osteoclastogenesis is not yet known.

Based on the partial amino acid sequence, cDNA for human OCIF was molecularly cloned. The amino acid sequence deduced from the nucleotide sequence of OCIF cDNA predicted that it consists of 401 amino acid residues, including a putative 21-amino acid residue signal sequence (5). The nucleotide sequence analysis has revealed that OCIF is identical to osteoprotegerin (6). OCIF has seven major domains (domains 1–7) and has overall similarity to proteins of the tumor necrosis factor receptor (TNFR) family, although OCIF lacks an apparent transmembrane region (5, 6). Domains 1–4 are cysteine-rich structures with a characteristic of extracellular domains of the TNFR family proteins. Domains 5 and 6 share structural features with "death domains" of TNFR 1, Fas, DR 3 (also designated as Apo 3, Wsl 1, and TRAMP), the TRAIL receptor, and the several recently identified cytoplasmic proteins mediating apoptosis (5, 7–17). However, unlike previously characterized death domains, two death domain homologous regions (DDHs), domains 5 and 6 of OCIF, exist in extracellular environments, because OCIF is secreted into conditioned medium. Domain 7, which does not resemble any protein motifs characterized thus far, consists of 50 amino acid residues and has a relatively high net positive charge; it contains eight basic amino acid residues (Lys and Arg) and only one acidic residue (Glu).

To determine which residue(s) or domain(s) is/are involved in the *in vitro* biological activity, binding to heparin, and dimer formation, we generated and characterized various mutants of OCIF. We also examined the potential of domains 5 and 6 for mediating cell death by overexpressing chimeric proteins in which portions containing the transmembrane domain derived from Fas were inserted into OCIF.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Cell Lines, and Culture—*Escherichia coli* DH5α (Life Technologies, Inc.) was used to propagate and amplify plasmids. 293-EBNA (CLONTECH), a human fetal kidney cell line, was grown in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS) and 250 μg/ml geneticin (Sigma). A mouse bone marrow-derived stromal cell line, ST2 (Riken Cell Bank RCB0224, Japan) was grown in minimum essential medium-α containing 10% FBS.

Construction of Plasmids and Expression of Mutants of OCIF—Mammalian expression plasmid pCEP4 (CLONTECH) was used for expression of OCIF mutants, Fas, and OCIF-Fas chimeric proteins. Full-length OCIF cDNA was subcloned into the *Xho*I and *Bam*HI sites of pCEP4 to yield pCEP4-OCIF in which the cDNA is expressed under the control of the cytomegalovirus promoter. Human Fas cDNA (18) was amplified by PCR using Ex *Taq* polymerase (Takara Shuzo) and primers 5′-TCTTTCACTTCGGAGGATTG-3′ (sense) and 5′-TCTAGACCAAGCTTTGGATTTC-3′ (antisense). A human activated T cell cDNA library (CLONTECH) was used as a template for the PCR. Mutagenesis and genetic fusions were performed according to a method called "recombinant polymerase chain reaction" (19). To generate the expression vector for OCIF mutants, DNA fragments amplified by PCR were digested with appropriate restriction enzymes (DNA fragments for ΔD1,

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[1] The abbreviations used are: OCIF, osteoclastogenesis inhibitory factor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; DDH, death domain homologous region; TRAP, tartaric-resistant acid phosphatase; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; IMDM, Iscove's modified Dulbecco's medium; FPLC, fast protein liquid chromatography; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; bp, base pair(s); kb, kilobase pair(s).

ΔD2, and ΔD3 by *XhoI/NdeI*; ΔD4 by *XhoI/SphI*; ΔD5, C195S, C202S and C277S by *NdeI/SphI*; ΔD6 and C319S by *NdeI/Bst*EII; ΔCL and C400S by *SphI/Bst*EII; ΔD7 by *SphI/Bam*HI; and ΔD67 and ΔD567 by *NdeI/Bam*HI) and were substituted for the corresponding region of OCIF cDNA in pCEP4-OCIF. The plasmids thus constructed were designated pCEP4-ΔD1, pCEP4-ΔD2, pCEP4-ΔD3, pCEP4-ΔD4, pCEP4-ΔD5, pCEP4-C195S, pCEP4-C202S, pCEP4-C277S, pCEP4-ΔD6, pCEP4-C319S, pCEP4-ΔCL, pCEP4-C400S pCEP4-ΔD7, pCEP4-ΔD67, and pCEP4-ΔD567, respectively. To construct a vector expressing OCIF-Fas, DNA fragment coding for domains 1–4 of OCIF (amplified using primers 5′-TGACAAATGTCCTCCTGGTA-3′ and 5′-AGATCTCGATCCATCTATTCCACATTTTTGAGTTG-3′) and that for the transmembrane domain through the intracellular region of Fas (amplified using primers 5′-TGTGGAATAGATGGATCGAGATCTAACTTGGGGTGGCTT-3′ and 5′-CCGGATCCTCTAGACCAAGCTTTGGATTTC-3′) were fused as described previously (19). The fused fragment was digested with *NdeI* and *Bam*HI, and the resultant fragment was substituted for the *NdeI/Bam*HI fragment of OCIF cDNA in pCEP4-OCIF to generate pCEP4-OCIF-Fas. To generate pCEP4-TM-OCIF, first, a DNA fragment encoding the transmembrane region of Fas was amplified by PCR with primers 5′-GGCTCGAGTCTTTCACTTCGGAGGATTG-3′ (sense) and 5′-CCTCCGGAACCTTGGTTTTCCTTTCTGTG-3′ (antisense), and the human activated T cell cDNA library as a template. Next, the amplified fragment was digested with *Bsp*EI, and the ~700-bp fragment produced was ligated with the 0.4-kb *Bsp*EI/*Sph*I fragment of OCIF cDNA. Then, the ligated DNA fragment was digested with *Bgl*II and *Sph*I, and a ~550-bp fragment was isolated. Finally, a ~700-bp *Bgl*II/*Xho*I fragment from pCEP4-OCIF-Fas, the ~550-bp fragment, and an ~11-kb *XhoI/SphI* fragment from pCEP4-OCIF were ligated. To construct pCEP4-TM-OCIFΔD567, a DNA sequence encoding domains 5 to 7 was deleted from pCEP4-TM-OCIF by recombinant PCR (19). Mutations were confirmed by DNA sequencing. For the production of ΔD1, ΔD2, ΔD3, ΔD4, ΔD5, ΔD6, ΔD7, ΔD67, ΔD567, ΔCL, C195S, C202S, C277S, C319S, and C400S, 293-EBNA cells were seeded on 24-well plates at a cell density of ~2×10^5/well, and the cells in each well were transfected with 1 μg of the respective expression vector on the following day using LipofectAMINE (Life Technologies, Inc.). Five hours after transfection, the DNA precipitate was removed, and the cells were cultured in serum-free medium for 40 h. Cells transfected with pCEP4-ΔD567 were cultured in IMDM containing 10% FBS and 200 μg/ml hygromycin B (Wako) to establish stable transformants.

Establishing ELISA to Quantify OCIF and OCIF Mutants—An ELISA employing rabbit anti-OCIF polyclonal antibody was used to quantify OCIF mutants in the conditioned medium. The polyclonal antibody was prepared as follows. OCIF produced in 293-EBNA harboring pCEP4-OCIF was purified to homogeneity as described previously (4). JW rabbits were immunized with the purified OCIF. Anti-OCIF antibody was purified from serum of the rabbits using protein G-Sepharose (Pharmacia Biotech Inc.). Horseradish peroxidase-labeled anti-OCIF antibody was prepared using a maleimide-activated peroxidase kit (Pierce).

Assay for Biological Activity of OCIF Mutants—Osteoclastogenesis inhibitory activity was determined by observing the suppression of osteoclast-like cell formation. Osteoclast-like cell formation was evaluated by measuring tartaric acid-resistant acid phosphatase (TRAP) activity in co-culture of mouse spleen cells and ST2 cells after cultivating for 1 week in the presence of 10 nM 1,25-dihydroxyvitamin D_3 and 100 nM dexamethasone concomitant with various concentrations of OCIF mutants as described previously (4). TRAP activity is expressed in absorbance at 405 nm as described previously (4).

Purification of ΔD567—Mutant ΔD567 was purified from conditioned medium of a stable 293-EBNA transfectant using rabbit anti-OCIF polyclonal antibody-immobilized HiTrap NHS-activated column (Pharmacia). The conditioned medium (~100 ml) was applied to the column at a flow rate of 0.5 ml/min. After washing the column with 21.5 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 M NaCl and 0.1% CHAPS (Sigma) and subsequently with 8 ml of 0.15 M NaCl containing 0.1% CHAPS, proteins were eluted from the column with 0.2 M acetic acid (pH 2.5) containing 0.15 M NaCl and 0.1% CHAPS at a flow rate of 0.5 ml/min. Fractions containing ΔD567 were determined by an ELISA employing anti-OCIF polyclonal antibody, collected, and applied to a HiTrap Blue (Pharmacia) column equilibrated with 10 mM phosphate buffer (pH 6.0) containing 0.1% CHAPS. After washing the column with the same buffer, proteins bound to the column were eluted with a linear gradient from 0 to 2 M NaCl at a flow rate of 0.5 ml/min. Fractions containing ΔD567 (~10 μg) were concentrated and desalted using Centricon 10 (Amicon).

Heparin-Sepharose Chromatography—The affinity of wild-type OCIF and OCIF mutants for heparin was determined by FPLC on HiTrap heparin column (Pharmacia). Conditioned medium (~1 ml) containing each OCIF mutant was applied to the column equilibrated with 50 mM Tris-HCl (pH 7.0) containing 0.1% CHAPS. The column was developed with a 40-min linear gradient of 0 to 1 M NaCl in equilibration buffer at a flow rate of 1.0 ml/min, and fractions (1.0 ml) were collected. The concentration of the mutant in each fraction was determined by ELISA employing rabbit anti-OCIF polyclonal antibody.

Western Blotting—Proteins were separated on SDS-polyacrylamide gel (10 or 13%) electrophoresis. Rainbow-colored molecular weight markers (Bio-Rad) were used as standards. Proteins were blotted onto ProBlott (Perkin-Elmer Corp.) using a semidry-type electroblotter (Bio-Rad). OCIF or OCIF mutants were detected using horseradish peroxidase (HRP)-conjugated rabbit anti-OCIF polyclonal antibody, and the membrane was exposed to x-ray film using enhanced chemiluminescence system (ECL, Amersham Corp.).

Reverse Transcriptase PCR—Total RNA was isolated from cells 24 h posttransfection using Trizol (Life Technologies, Inc.). Reverse transcriptase-PCR was performed with Super Script preamplification system (Life Technologies, Inc.) using 1 μg of the total RNA. Primers for the reverse transcriptase-PCR were 5′-ATGAACAACTTGCTGTGCTGCGCGCT-3′ (sense) and 5′-CAAACTGTATTTCGCTCTGG-3′ (antisense). The primers were designed to amplify a 424-bp fragment corresponding to the putative signal peptide and domains 1–3 of OCIF. Size of the amplified products was determined by 1% agarose gel electrophoresis.

Detection of OCIF Antigen Expressed on Cell Surface—293-EBNA cells were inoculated into each well in a 96-well plate at a cell density of ~2×10^4 cells/200 μl/well. The cells in each well were transfected on the following day with 250 ng of empty vector, pCEP4-Fas, pCEP4-OCIF-Fas, pCEP4-TM-OCIF, or pCEP4-TM-OCIFΔD567 using LipofectAMINE. Twenty hours after transfection, cells were fixed with 1% glutaraldehyde (Wako) for 10 min at room temperature. After washing the cells twice with 200 μl of phosphate buffered saline (PBS), HRP-conjugated anti-OCIF polyclonal antibody was added to the wells, and the plate was incubated for 2 h at room temperature. After washing the cells five times with 200 μl of PBS, 100 μl of substrate solution (0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) and 0.006% H_2O_2 in 0.1 M citrate-phosphate buffer, pH 4.5) was added to the cells, and the incubation was continued for additional 3 min at room temperature. The reaction was stopped by adding 50 μl of 6 N H_2SO_4 to each well, and the absorbance at 490 nm was measured.

Cell Death Assays—293-EBNA cells were inoculated into each well in a 24-well plate at a cell density of ~5×10^4 cells/2 ml/well. The cells in each well were transfected on the following day with 1 μg of empty vector, pCEP4-Fas, pCEP4-OCIF-Fas, pCEP4-TM-OCIF, or pCEP4-TM-OCIFΔD567, together with 0.2 μg of pCH110, an expression plasmid for β-galactosidase (Pharmacia), using LipofectAMINE. Five hours after transfection, the DNA precipitate was removed, and IMDM containing 10% FBS (500 μl/well) was added to each well. After 15 h of cultivation, cells were fixed by treating with 1% glutaraldehyde for 5 min at room temperature, washed twice with 1 ml of PBS, and stained with X-gal (Wako). Cell morphology was examined under a phase-contrast microscope, and the percentage of round-shaped blue cells *versus* total blue cells was calculated. For the assay of lactate dehydrogenase activity in the medium, aliquots of conditioned medium were removed before fixing the cells. Lactate dehydrogenase activity was measured using a colorimetric kit (Shino-test, Tokyo, Japan). DNA fragmentation assay was performed as described previously (20) using cells cultivated for 20 h after transfection.

RESULTS

Construction of Vectors and Expression of OCIF Mutants—A schematic representation of OCIF and mutants of OCIF used in this work is shown in Fig. 1. We constructed a series of expression vectors and transfected them into 293-EBNA cells to produce deletion mutants, ΔD1, ΔD2, ΔD3, ΔD4, ΔD5, ΔD6, and ΔD7. We also constructed expression vector for C-terminal truncation mutants, ΔD67 and ΔD567. Furthermore, to identify a residue responsible for dimer formation of OCIF, a series of Cys to Ser mutants, C195S, C202S, C277S, C319S, and C400S, in which each Cys residue in domains 5, 6, and 7 was replaced with Ser, and a deletion mutant, ΔCL, which lacks two C-terminal amino acid residues, Cys-400 and Leu-401, were prepared. All but two mutants (ΔD1 and ΔD2) were detected in

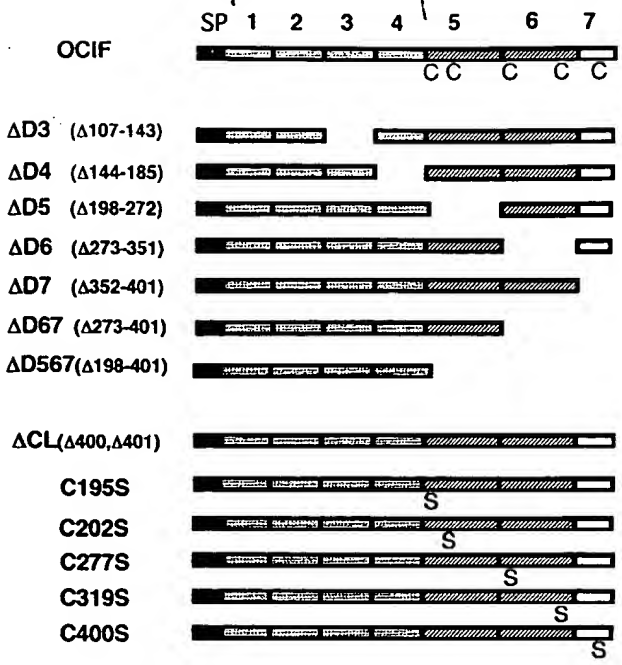

FIG. 1. **Schematic representation of OCIF mutants.** *SP*, signal peptide; *C*, cysteine residues in domains 5–7; *S*, positions of Cys to Ser substitutions. Numbers on the *top* indicate the domains. Amino acid residues deleted in each mutant are indicated in *parentheses*.

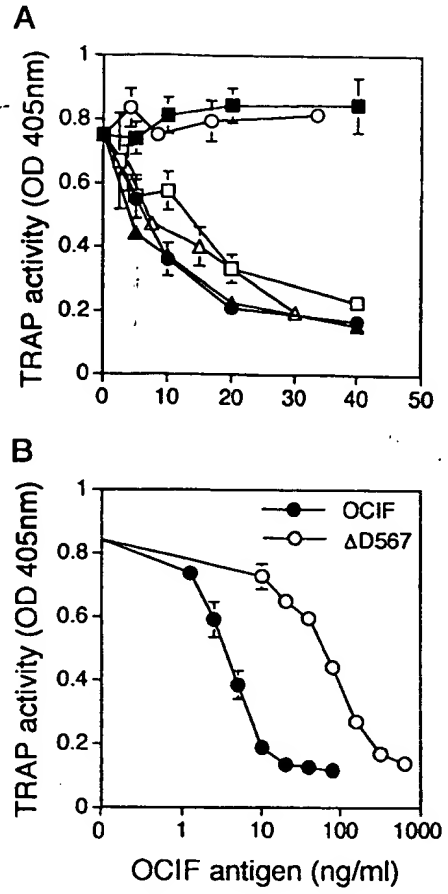

FIG. 2. **Osteoclastogenesis inhibitory activity of OCIF deletion mutants.** Inhibition by OCIF mutants of osteoclast-like cell formation in the co-cultures was evaluated by measuring TRAP activity. Co-cultures of ST2 and mouse spleen cells were incubated with various concentrations of the OCIF mutants. Data are expressed as mean ± SD of triplicate experiments. *A*, OCIF antigen in the supernatant of cells transiently expressing each mutant was quantified by an ELISA employing rabbit anti-OCIF polyclonal antibody. ●, OCIF; ○, ΔD3; ■, ΔD4; □, ΔD5; ▲, ΔD7; and △, ΔD67. *B*, mutant ΔD567 was purified to homogeneity, and the purified protein was used for the assay. ●, OCIF; ○, ΔD567.

the conditioned medium by Western blot analysis (see below) employing anti-OCIF polyclonal antibody. OCIF mutants were quantified by ELISA using anti-OCIF polyclonal antibody. ΔD1 and ΔD2 were not secreted at levels detectable by Western blotting or ELISA. The concentration of most of the mutants in their conditioned media ranged from 200 ng/ml to 2 μg/ml. Accurate determination of inhibitory activity of ΔD6 was impossible due to poor productivity (~50 ng/ml).

The N-terminal Portion of OCIF Containing Domains 1–4 Is Sufficient to Inhibit Osteoclastogenesis—Fig. 2*A* shows the osteoclastogenesis inhibitory activity of the deletion mutants measured based on the inhibition of TRAP-positive osteoclast-like cell formation in the cocultures. Although wild-type OCIF inhibited the osteoclast-like cell formation in a dose range of 5 to 40 ng/ml (half-maximal inhibitory dose (ID_{50}) of ~6 ng/ml), ΔD3 and ΔD4 failed to inhibit the osteoclast-like cell formation at concentrations 35 and 40 ng/ml, respectively. In contrast, ΔD5 retained the inhibitory activity with an ID_{50} of ~15 ng/ml. Furthermore, ΔD7 had a specific activity comparable to that of wild-type OCIF. A C-terminal truncation mutant ΔD67, which lacks domains 6 and 7, possessed the osteoclastogenesis inhibitory activity, although potency in the inhibitory activity was considerably lower than that of wild-type OCIF (an ID_{50} of 10 ng/ml) (Fig. 2*A*). For the accurate determination of the inhibitory activity of ΔD567, which lacks DDHs entirely, we purified it using an anti-OCIF-antibody-immobilized affinity column. The purified ΔD567 inhibited osteoclastogenesis (Fig. 2*B*), indicating that truncation of domains 5–7 (consisting of the C-terminal 204 residues) does not abolish the biological activity. However, the potency of ΔD567 was approximately 10% of that of wild-type OCIF (Fig. 2*B*) as estimated from their ID_{50}. These results indicate that the N-terminal portion containing the first four domains is sufficient for exerting the osteoclastogenesis inhibitory activity *in vitro*.

Deletion of Domain 7 Decreases the Affinity of OCIF for Heparin—To examine the significance of the binding of OCIF to heparin in the inhibition of osteoclastogenesis, we analyzed the affinity of ΔD7, ΔD67, and ΔD567 for heparin by FPLC on HiTrap heparin column. Conditioned medium of the cells transiently expressing each OCIF mutant was loaded on the column and each mutant was eluted from the column with NaCl-containing buffer. Wild-type OCIF was eluted at NaCl concentrations of 0.55 M and 0.74 M, which correspond to those at which the monomer and the dimer form of OCIF are eluted, respectively (Fig. 3). ΔD7 was eluted as a single peak at an NaCl concentration of 0.24M. Further truncation had only marginal effects on the binding of OCIF to heparin (Fig. 3). These three mutant proteins were eluted as single peaks, probably because they are present as monomers (see below). These results strongly suggest that domain 7, which occupies the C-terminal 50 amino acid residues, contains a heparin-binding site. The fact that deletion of domain 7 did not affect the inhibition of osteoclastogenesis (Fig. 2*A*) but significantly decreased the binding of OCIF to heparin (Fig. 3) indicates that binding ability of OCIF to heparin does not correlate with its osteoclastogenesis inhibitory activity *in vitro*.

Domain 7 Is Involved in the Dimerization of OCIF—OCIF from IMR-90-conditioned medium is present as two forms, a monomer with an approximate molecular mass of 60 kDa and a disulfide-linked dimer with an approximate mass of 120 kDa (4). To identify (*a*) domain(s) responsible for the dimer formation, the capability of the domain deletion mutants to form dimers was analyzed using immunoblotting as shown in Fig. 4*A*. A protein with a mass of 80–100 kDa was detected for ΔD3,

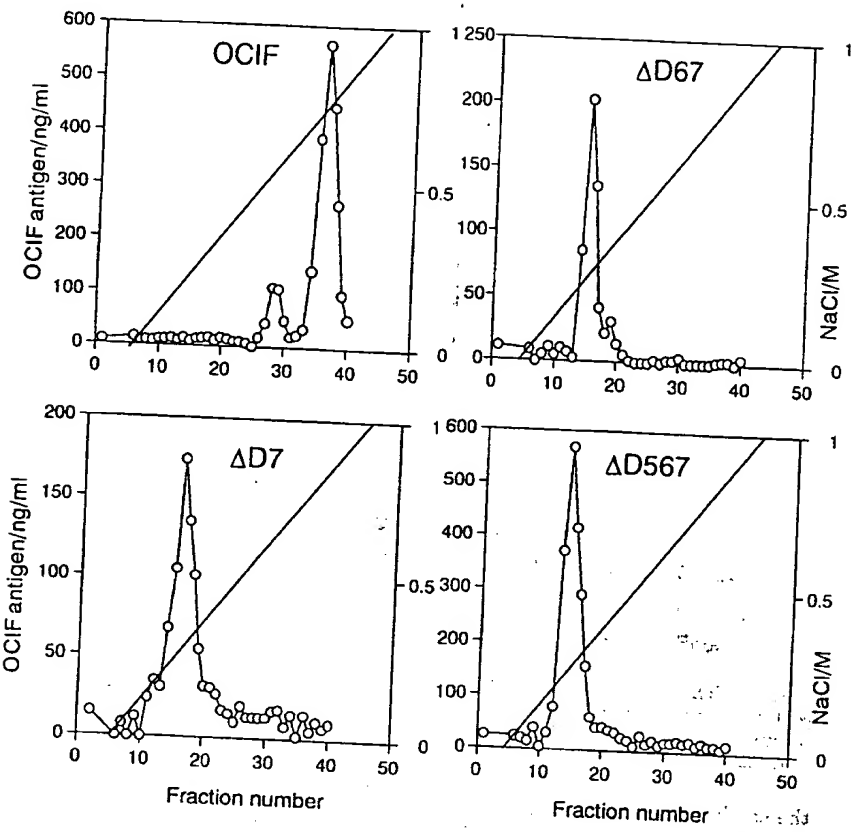

FIG. 3. **Heparin-affinity HPLC profiles of the OCIF mutants with C-terminal truncation.** Conditioned medium (1 ml) of cells transiently expressing each mutant was applied to a heparin-affinity FPLC column and each mutant was eluted from the column with a linear gradient of NaCl (0–1 M). Fractions were assayed for OCIF using ELISA.

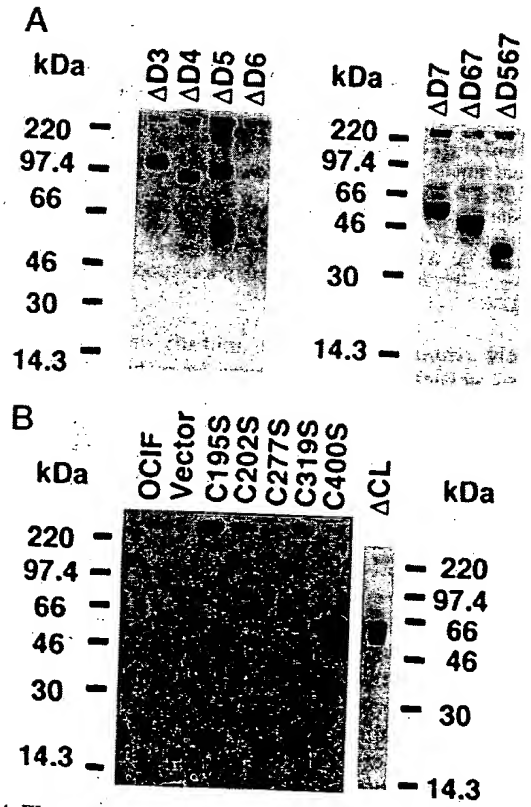

FIG. 4. **Western blot analysis of OCIF deletion mutants.** Conditioned medium (10 μl) containing each mutant was subjected to SDS-PAGE (10 or 13% acrylamide). The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated with HRP-labeled anti-OCIF polyclonal antibody. The molecular mass standards are myosin (220 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). *A*, analysis of OCIF deletion mutants; *B*, analysis of mutants C195S, C202S, C277S, C319S, C400S, and ΔCL.

ΔD4, ΔD5, and ΔD6 as a major band. In contrast, ΔD7 is present almost exclusively as a ~55-kDa protein. Thus, ΔD7 is present mainly as a monomer, while ΔD3, ΔD4, ΔD5, and ΔD6 are present in two forms, a monomer and a dimer (or a multimer) in the conditioned medium, suggesting that domain 7 is involved in the dimer formation. ΔD67 and ΔD567, both lacking domain 7, migrated as monomers as expected (Fig. 4*A*). Difference in size between ΔD3 and ΔD4 (Fig. 4*A*) is probably due to a different degree of glycosylation. Indeed, there are three potential *N*-glycosylation sites (Asn-*X*-Ser/Thr) in domain 3, whereas there is no such site in domain 4 (5, 6).

Cys-400 Is Responsible for the Dimer Formation—Since there is only one Cys residue (Cys-400) in domain 7 (5, 6), participation of the residue in the intermolecular disulfide-linkage was suspected. To confirm this, two mutants, one with substitution of a Ser residue for Cys-400 (C400S) and the other with a deletion of the two C-terminal amino acid residues Cys-400 and Leu-401 (ΔCL) were produced. As a control, a series of Cys to Ser mutants, C195S, C202S, C277S, and C319S, in which each Cys residue in domains 5 and 6 was replaced with a Ser residue, were generated. The mutants were transiently expressed in 293-EBNA cells, and the structure of the mutants was analyzed by Western blotting as shown in Fig. 4*B*. The results indicate that both C400S and ΔCL exist almost exclusively as a monomer with a mass of ~60 kDa (Fig. 4*B*). No monomer-form OCIF with a mass of ~60 kDa was detected in the conditioned medium of C195S, C202S, C277S, or C319S (Fig. 4*B*). These four mutants migrated even slower than the dimer form OCIF with a mass of ~120 kDa. The slower migrating bands may represent higher order multimers derived from unusual disulfide bonding. These results demonstrate that Cys-400 is responsible for the dimer formation of OCIF. Thus, we conclude that the 120-kDa protein detected in the conditioned medium of OCIF-producing cells is a homodimer consisting of two 60-kDa monomers linked together by an intermolecular disulfide bond between two Cys-400 residues.

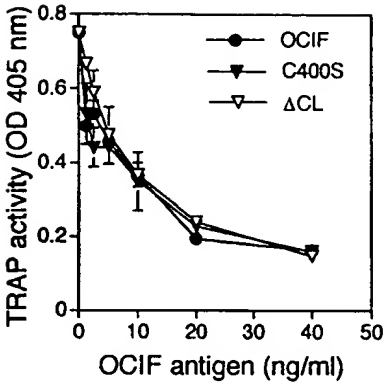

FIG. 5. **Osteoclastogenesis inhibitory activity of OCIF mutants, C400S, and ΔCL.** Inhibition by OCIF mutants of osteoclast-like cell formation was determined as described under "Experimental Procedures" and in the legend to Fig. 2. OCIF antigen in the supernatant of cells transiently expressing each mutant was quantified by an ELISA employing rabbit anti-OCIF polyclonal antibody.

C400S and ΔCL, which are present almost exclusively as a monomer in the conditioned medium, were as potent as wild-type OCIF in the inhibition of the *in vitro* osteoclast formation (Fig. 5). These results provide further evidence that formation of the dimer is not essential for exerting the *in vitro* osteoclastogenesis inhibitory activity. C195S, C202S, C277S, and C319S, which are present mainly as multimers as shown in Fig. 4*B*, maintained the biological activity (data not shown).

Overexpression in 293-EBNA Cells of Chimeric Proteins Consisting of OCIF and the Fas Transmembrane Domain Causes Apoptosis—We next asked whether domains 5 and 6 (DDHs) have a potential for mediating cytotoxic signals. For this purpose, we transfected plasmids encoding various OCIF/Fas fusion proteins together with pCH110, an expression plasmid for β-galactosidase, in 293-EBNA cells. The structure of OCIF, Fas, and their fusion proteins used in this experiment is schematically illustrated in Fig. 6*A*. The presence of mRNA derived from each chimeric construct in the transfected cells was confirmed by reverse transcriptase-PCR. Primers were designed to amplify a 424-bp fragment corresponding to the 5′ portion of OCIF mRNA. As shown in Fig. 6*B*, reverse transcriptase-PCR using RNA from the cells transfected with pCEP4-OCIF-Fas, pCEP4-TM-OCIF, or pCEP4-TM-OCIFΔD567 generated the 424-bp fragment, but not with pCEP4 or pCEP4-Fas. HRP-labeled anti-OCIF polyclonal antibody specifically bound to the cells transfected with pCEP4-OCIF-Fas, pCEP4-TM-OCIF, or pCEP4-TM-OCIFΔD567, but not with the empty vector or pCEP4-Fas (Fig. 6*C*). These results indicate that each chimeric cDNA was efficiently expressed, and the fusion products were translocated to the surface of the transfected cells. These cells were then stained with X-gal to examine the size and the shape of the cells harboring each expression plasmid. Microscopic examination of the cells transfected with pCEP4-Fas, pCEP4-OCIF-Fas, or pCEP4-TM-OCIF revealed that 30–60% of the blue cells were round and shrunken, showing signs of cell death (Fig. 7*A*). In contrast, when transfected with the empty vector, pCEP4-OCIF or pCEP4-TM-OCIFΔD567, more than 90% of the blue cells retained the flat and adherent appearance (Fig. 7*A*). Lactate dehydrogenase activity in the conditioned medium of the cells transfected with pCEP4-OCIF-Fas or pCEP4-TM-OCIF was significantly higher than that transfected with the empty vector or pCEP4-TM-OCIFΔD567 (Fig. 7*B*), showing that overexpression of OCIF-Fas or TM-OCIF induced cell death. The transfection efficiency was almost the same (approximately 40%) in all transfection experiments. Cytotoxic signal induced by OCIF-Fas was apparently stronger than that induced by Fas for a currently unknown reason. To examine

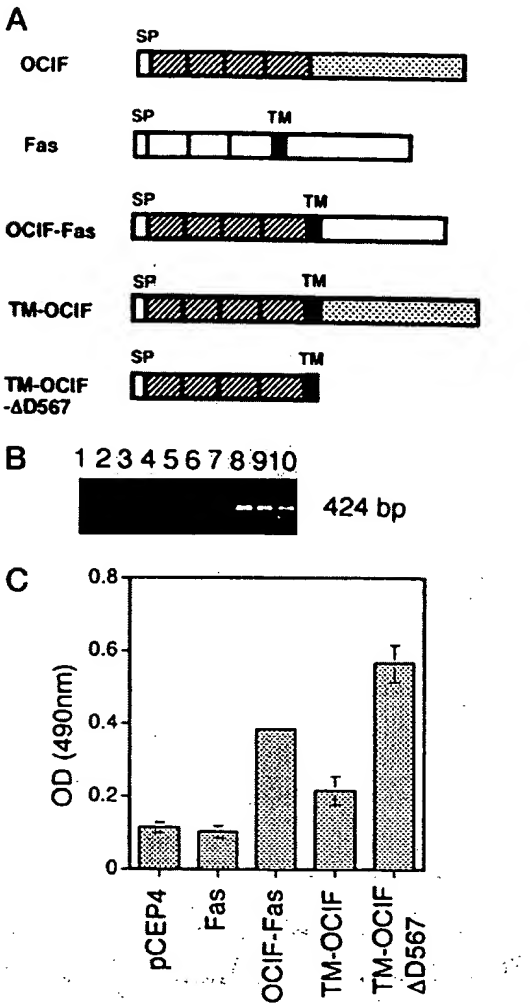

FIG. 6. **Expression of chimeric receptors.** *A*, schematic representation of chimeric receptors. *Hatched boxes*, cysteine-rich regions of OCIF; *dotted boxes*, domains 5–7 of OCIF; *open boxes*, extracellular and intracellular domains of Fas; *closed boxes*, transmembrane domain of Fas; *SP*, signal sequence. *B*, detection of mRNA for the OCIF/Fas chimeric protein in 293-EBNA cells transfected with each expression plasmid. First strand cDNA was prepared from total RNA extracted from 293-EBNA cells transfected with each plasmid and was subjected to PCR with the primers described under "Experimental Procedures." The PCR products were separated on an 1% agarose gel. cDNA synthesis reactions were performed without (*lanes 1–5*) or with (*lanes 6–10*) reverse transcriptase. PCR products derived from cells transfected with pCEP4 (*lanes 1* and *6*), with pCEP4-Fas (*lanes 2* and *7*), with pCEP4-OCIF-Fas (*lanes 3* and *8*), with pCEP4-TM-OCIF (*lanes 4* and *9*), or with pCEP4-TM-OCIFΔD567 (*lanes 5* and *10*) are shown. *C*, detection of OCIF antigen on the surface of 293-EBNA cells transfected with the plasmid that expresses OCIF/Fas chimeric protein. 293-EBNA cells (~2 × 10^4/well) were seeded on a 96-well plate. The cells in each well were transfected on the following day with 250 ng of either the empty vector or each expression plasmid using LipofectAMINE. Twenty hours after transfection the cells were fixed with glutaraldehyde and washed twice with PBS. The OCIF antigen on the cells was detected by HRP-conjugated anti-OCIF polyclonal antibody as described under "Experimental Procedures." Data are expressed as mean ± S.D. of triplicate experiments.

whether the cell death was caused by apoptosis, we next analyzed the integrity of DNA in the cells transfected with the empty vector, pCEP4-OCIF-Fas or pCEP4-TM-OCIF. Cells transfected with pCEP4-OCIF-Fas or pCEP4-TM-OCIF showed severe fragmentation of DNA, a clear symptom of apoptosis, as compared with controls (Fig. 7*C*). These results demonstrate that OCIF is capable of triggering apoptosis when the Fas transmembrane region is inserted between cysteine-rich regions and DDHs and

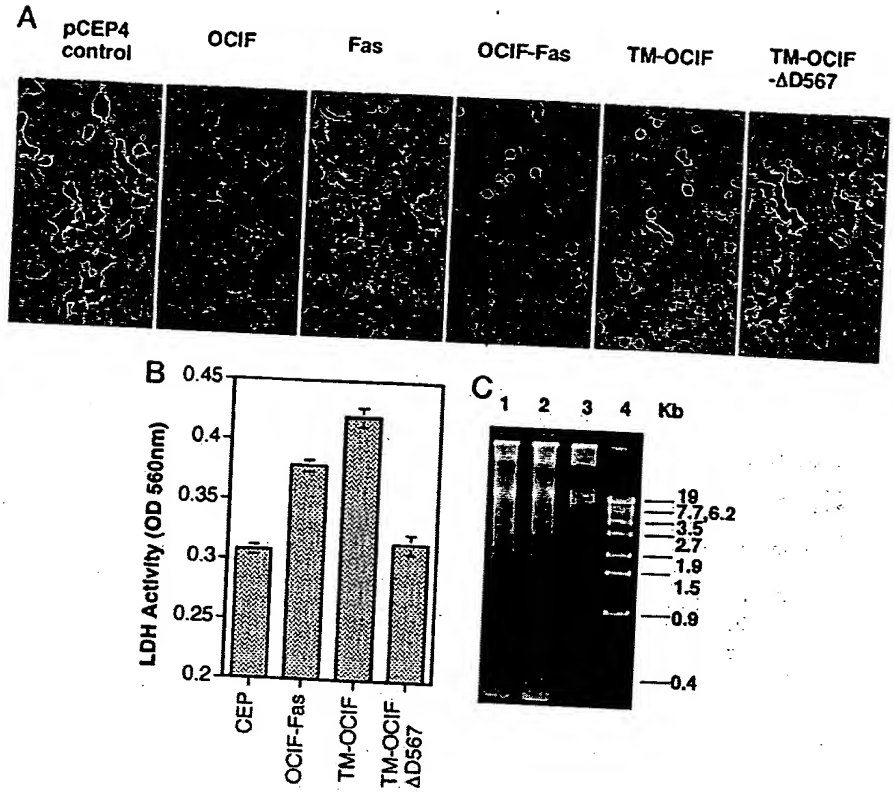

FIG. 7. **Induction of cytotoxic signal by overexpression of TM-OCIF on 293-EBNA cells.** *A*, morphology of 293-EBNA cells transfected with expression vectors. Cells (~4 × 10^4 cells/well) on a 24-well plate were transfected with the indicated expression vector (1 μg) plus pCH110 (0.2 μg). After 20 h, the cells were fixed, stained with X-gal, and photographed under a phase contrast microscope. *B*, lactate dehydrogenase (*LDH*) released in the medium. Cells were transfected as in *A*, and 50 μl of the conditioned medium were subjected to the assay for lactate dehydrogenase activity. Data are expressed as mean ± S.D. of triplicate experiments. *C*, DNA fragmentation in transfected 293-EBNA cells. Cells (~6 × 10^5 cells/well of a 6-well plate) were transfected with the indicated expression vectors. Twenty hours after transfection, cellular DNA was isolated and the whole DNA preparation was applied on a 2% agarose gel as described in Hartmann *et al.* (20). *Lane 1*, DNA from pCEP4-OCIF-Fas-transfected cells; *lane 2*, DNA from pCEP4-TM-OCIF-transfected cells; *lane 3*, DNA from pCEP4-transfected cells; *lane 4*, size markers.

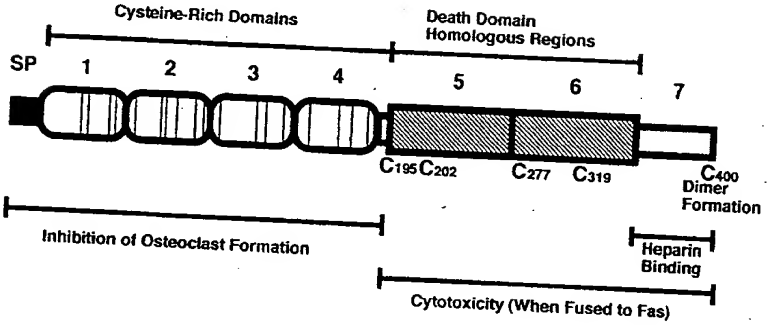

FIG. 8. **Representation of functional domains in OCIF.** *Closed box*, signal sequence (*SP*); *open boxes* with *vertical bars*, cysteine-rich domains with cysteine residues; *hatched boxes*, death domain homologous regions; *open square box*, domain 7; *C*, cysteine residues in domains 5–7.

that domains 5–7 have a potential comparable to the death domain of Fas in the induction of apoptosis.

DISCUSSION

OCIF belongs to the TNFR family, containing four cysteine-rich domains and two DDHs followed by a domain with a high net positive charge (5, 6). In addition, OCIF has some characteristics that not all of the TNFR family proteins possess: (i) it is a secretory protein with no apparent transmembrane region; (ii) it is present in two forms, a monomer and a dimer; and (iii) it interacts with heparin. In the present study, we examined which structural domains are involved in the inhibition of osteoclastogenesis, the binding to heparin, and the formation of the dimer. We also examined whether the two DDHs have potential for mediating the cytotoxic signal when a chimeric protein in which the Fas transmembrane region is inserted in front of DDHs is produced in cells. The results are summarized in Fig. 8.

By analyzing the osteoclastogenesis inhibitory activity of deletion and C-terminal truncation mutants, we found that the N-terminal portion containing domains 1–4 is sufficient to inhibit osteoclastogenesis *in vitro*. Domains 1–4 correspond to the extracellular cysteine-rich regions of the TNFR family proteins. For the TNFR family proteins, these regions protrude outside the cells and are involved in the interaction with their ligands. Even for the soluble form receptors, the cysteine-rich regions still have the ability to associate with their ligands. A naturally occurring secreted form of Fas lacking the transmembrane domain is present in patients with systemic lupus erythematosus, suggesting that the molecule works as an antagonist against Fas (22). A soluble form of TNFR consisting of the cysteine-rich region, which is generated by a proteolytic cleavage, inhibits the activity of both TNF-α and TNF-β (23–26). Thus, most of the soluble variants of the TNFR family proteins act as antagonists against the membrane-bound signaling receptors. This fact raises the possibility that OCIF competes with a currently unidentified receptor capable of triggering osteoclast formation, by binding to a ligand with a structural similarity to TNF (4).

Recently, Simonet *et al.* (6) have reported the isolation of a cDNA coding for osteoprotegerin, a protein identical to OCIF. They showed that the first four domains alone can exert its inhibitory activity *in vitro* by analyzing the biological activity of C-terminal truncation mutants (6). However, the biological activity of the mutants was not accurately determined in their study. We first found that conditioned medium of cells producing ΔD567 was capable of inhibiting osteoclastogenesis in a dose-dependent manner (data not shown). Then, the mutant was purified to homogeneity to determine the potency. By

analyzing the *in vitro* biological activity of the mutant, we concluded that the N-terminal portion of OCIF is sufficient to inhibit osteoclastogenesis, although the potency is approximately one tenth of that of wild-type OCIF (Fig. 2*B*).

Analysis of heparin binding capability of the mutants revealed that domain 7 is involved in heparin binding. Binding to heparin or heparin-like molecules is known to be important for such growth factors as basic fibroblast growth factor to function *in vitro* and *in vivo* (21). The affinity of OCIF for heparin, however, did not correlate with the *in vitro* biological activity. For some proteins, changes in heparin-binding capability affect stability, rate of clearance, and target cell specificity *in vivo*. For example, a point mutation in superoxide dismutase that caused reduction of affinity for heparin results in a 10-fold increase in its plasma concentration without affecting the specific enzymatic activity *in vitro* (27). Therefore, it is worth examining the possibility that affinity of OCIF for heparin may be of some physiological importance *in vivo*. Most of heparin binding sites of known growth factors consist of a cluster of basic amino acid residues (28). Although no apparent cluster of positively charged amino acid residues is present in domain 7, application of Edmundson's wheel model to residues 361–378 shows an α-helix in which Lys-361, Lys-368, Lys-369, Arg-372, and His-375 exist on one side of the helix and hydrophobic residues such as Leu, Val, Ile, or Phe on the opposite side (data not shown). Such localized basic residues may contribute to the binding of OCIF to heparin. This was supported by the results that the mutants with alanine substitutions for Lys-368, Lys-369, and Arg-372 in OCIF had a marked decrease in affinity for heparin.[2]

Domain 7 is also responsible for the dimerization of OCIF. This finding was derived from Western blot analysis of the mutants (Fig. 4*A*). Subsequent study identified Cys-400 as the residue essential for the dimer formation (Fig. 4*B*). Substitution or deletion of Cys-400 did not affect the activity of OCIF (Fig. 5), confirming the previous observation that both the monomer and the dimer form OCIF have similar specific activity in inhibition of *in vitro* osteoclastogenesis (4). The dimerization by disulfide bridges is extremely important for other TNF receptor homologs to exert inhibitory activity. Myxoma virus T2 protein, a TNFR homolog, is secreted as both a monomer and a dimer, and they bind to rabbit TNF-α with a similar affinity. Interestingly, the dimer is a more potent TNF inhibitor (29). The significance of dimer formation of OCIF remains open for the further investigation.

Domains 5 and 6 have a homology to death domains that are involved in transmitting apoptotic signals in many cells. Domains 5 and 6 are not essential for the inhibitory activity (Fig. 2, *A* and *B*), heparin binding (Fig. 3), or the dimerization (Fig. 4*A*), although deletion of both domains resulted in a marked decrease in *in vitro* biological activity (Fig. 2*B*). Therefore, we examined whether the two domains have a potential for mediating "death" signals when expressed in the cytoplasm of 293-EBNA cells. It has been reported that ectopic expression of death domain-containing receptor proteins including TNFR 1, Fas, DR3 (also known as Apo-3, Wsl-1, or TRAMP) and the TRAIL receptor lead to cell death via apoptosis (11–14). Although overexpression of OCIF does not cause cell death, a receptor type OCIF (TM-OCIF), which contains the transmembrane region of Fas between domains 4 and 5 of OCIF, possessed an ability to induce apoptosis in 293-EBNA cells (Fig. 7, *A–C*). Truncation of the DDHs prevented the cell death, strongly suggesting that domains 5 and/or 6 have a high potential for mediating the apoptosis. Analysis of the OCIF gene failed to identify any sequences encoding a potential membrane-spanning domain, suggesting that OCIF exists only in soluble form.[3] Therefore, it is unlikely that OCIF triggers apoptosis in a manner similar to TNFR 1 or Fas. Instead, OCIF may induce apoptosis in an unknown fashion under certain conditions.

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